

MICROBIAL HEAT PRODUCTION

A study of its analytical application

Paul Monk



Lund 19 80

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The analytical application of <u>microcalorimetry</u> to the investigation of <u>microbial metabolism</u> has been studied using several <u>lactic acid bacteria</u>. <u>Microcalorimetry</u> has been applied to the investigation of <u>glucose catabolism</u> by cell suspensions of <u>Streptococci</u> in the presence of <u>2,4-dinitrophenol</u>. The interpretation of <u>power-time curves</u> for aerobic growth of <u>S. lactis</u> in complex medium is discussed. The effect of <u>inoculum preparation</u> and its storage at low temperature, <u>medium composition</u> and the presence of oxygen on the growth of cultures has been evaluated. The observations are applied to the preparation of inoculum and culture medium suitable for the <u>identification</u> of several lactic acid bacteria. Simple <u>mixed cultures</u> have been investigated for <u>compatibility</u> and <u>stability</u> on repeated sub-culture.			
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This review is based on the following papers which will be referred to by the Roman numerals I - IX

- I MONK, P. and I. WADSÖ. 1968. A flow micro-reaction calorimeter. Acta. Chem. Scand. 22: 1842-1852.
- II DELIN, S., P. MONK, and I. WADSÖ. 1969. Flow micro-calorimetry as an analytical tool in microbiology. Science Tools. 16: 22-24.
- III MONK, P., and I. WADSÖ. 1975. The use of microcalorimetry for bacterial classification. J. Appl. Bact. 38: 71-74.
- IV MONK, P., W. FORREST, and I. WADSÖ. 1977. Calorimetric studies of lactic acid bacteria and the affect of 2,4-dinitrophenol on their catabolic regulation, p. 150-155. In I. Lamprecht, and B. Schaarschmidt (eds.), Application of calorimetry in life sciences. W. de Gruyter, Berlin.
- V FUJITA, T., P. MONK, and I. WADSÖ. 1977. Calorimetric identification of several strains of lactic acid bacteria. J. Dairy Res. 45: 457-463.
- VI MONK, P. 1978. Microbial calorimetry as an analytical method. Proc. Biochem. V13, No. 12.
- VII MONK, P.R. 1978. Aerobic growth thermograms of Streptococcus lactis obtained with a complex medium containing glucose. J. Bacteriol. 135: 373-378.
- VIII MONK, P. 1979. Thermograms of Streptococcus thermophilus and Lactobacillus bulgaricus in single and mixed culture in milk medium. J. Dairy Res. 46: 485-496.
- IX MONK, P.R. 1979. Catabolism of glucose by Streptococcus faecalis and Streptococcus agalactiae in the presence of 2,4-Dinitrophenol, anaerobically and aerobically as affected by buffer pH. In manuscript.

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1. INTRODUCTION

The measurement of heat production is a general way of observing microbial metabolism. The energy required by the cell to perform useful work is provided from the change in Gibbs energy between the reactants degraded and the products formed. The enthalpy change observed by calorimetry is the sum of the contributions from all the reactions which occur, catabolism of substrates, biosynthesis, maintenance and transport processes of many compounds across the cell wall.

The energy requirements of a living cell is almost entirely mediated through one compound, adenosine-triphosphate (ATP), which is the bridge between the energy yielding processes of catabolism and the energy demands of anabolism. One of the more simple methods of obtaining energy from the environment is fermentation, life without oxygen, where organic compounds serve as both electron donors and electron acceptors.

Among those organisms which rely upon fermentation to provide their energy requirements are the lactic acid group of bacteria, where glucose is degraded to essentially lactic acid by the glycolytic pathway yielding a net gain of two moles of ATP for each mole of glucose consumed. In the presence of oxygen further energy may become available depending upon the fate of the final compound of the glycolytic sequence, pyruvic acid.

The observation that growing microorganisms can have characteristic profiles for the rate of heat production (1,2,3, paper II) has generated much interest in the possibility of using these profiles to identify not only genera, but species. The reliable use of this novel technique depends upon a better understanding of energy coupling and its regulation to allow sensible experimental modification of the growth environment.

As the anabolic reactions contribute little to the observed heat production (4,5), emphasis must be placed on catabolism in an attempt to modify and control heat production. Power - time

curves^{*)} might then be specific for the metabolic capabilities of individual organisms. With this information both natural and industrial fermentations may yield knowledge about microbial growth and become amenable to regulation and control using the heat produced during growth.

The main aim of the work contained in this thesis has been to contribute to the development of suitable instrumentation, to understand some of the processes producing power-time curves, to modify them experimentally and apply them to areas of analytical importance. For this purpose representative strains of lactic acid bacteria have been chosen for study because of their relatively simple metabolism and fastidious nutritional requirements resulting from a limited synthetic ability.

2. INSTRUMENTATION

2.1. General principles

The design requirements of a calorimeter are determined by the quantity of heat to be measured, the properties of the reaction to be studied and the amount of material available. A balance must be made between the heat evolved and the sample size to minimize the time constants associated with samples of large heat capacity so that the record of heat production closely corresponds to the reaction occurring in the calorimetric vessel. For microbiological systems the heat evolved and the amount of sample available are both usually large, which allows instrumentation of high sensitivity to use small sample volumes. However, growth of microbial cultures, a rate process, is slow and the calorimeter must be stable for extended periods of time. This is satisfied by

^{*)} It is now recommended that power-time curve replace the commonly used term "thermogram" for the record of heat production. (Ref. Calorimetric Measurements on Cellular Systems. Recommendations for Measurements and Presentation of Results. Interunion Commission on Biothermodynamics, Oct. 1979.)

using twin systems where a differential signal is recorded and long-term drifts are mainly cancelled. Facilities must be incorporated for stirring or mixing, gas phase control, sterilization and the addition of reagents. To be useful as a general analytical method the technique must be rapid, simple to operate and have a minimum time for temperature equilibration between samples. The design and operation of many instruments has been reviewed (6).

2.2. Flow and ampoule calorimeters

The two calorimeters used in this work were both of the heat conduction type. A batch calorimeter (7) was modified to a flow system and its properties and method of operation are described in paper I.

The heat liberated in the reaction vessel is transferred through thermoelectric elements positioned on either side of the vessel to a heat sink which is maintained at a constant temperature by a thermostated water bath. The temperature difference across the thermopile generates a voltage signal V , which is proportional to the heat flow, $\frac{dq}{dt}$, where

$$\frac{dq}{dt} = \epsilon \cdot V \quad [1]$$

For steady state reactions the heat flow is proportional to the power evolved,

$$P = \epsilon \cdot V \quad [2]$$

With a flow calorimeter steady state heat production can be obtained by mixing two reactants on entering the calorimetric vessel or with reactions which are inherently steady state as found with substrate saturated solutions of enzymes (8). Washed cell suspensions of bacteria catabolizing a substrate also proceed at a constant rate and this has been applied to measuring the catabolism of glucose by *Streptococci* reported in papers IV and IX.

Growth of bacteria is a variable process and the power -

time curve recorded can be distorted due to large instrumental time constants. However, when the change in heat production is slow a good approximation of the relation [2] is found. No correction of the power-time curves were made for the growth of bacterial cultures studied.

The arrangement of an ampoule calorimeter (9) is shown in Fig. 1. The sample to be examined is contained in a Teflon coated stainless steel ampoule of about 1 ml which is closed with a screw lid and O-ring. The method of obtaining and recording the power-time curve of the reaction is similar to the flow calorimeter, paper I.

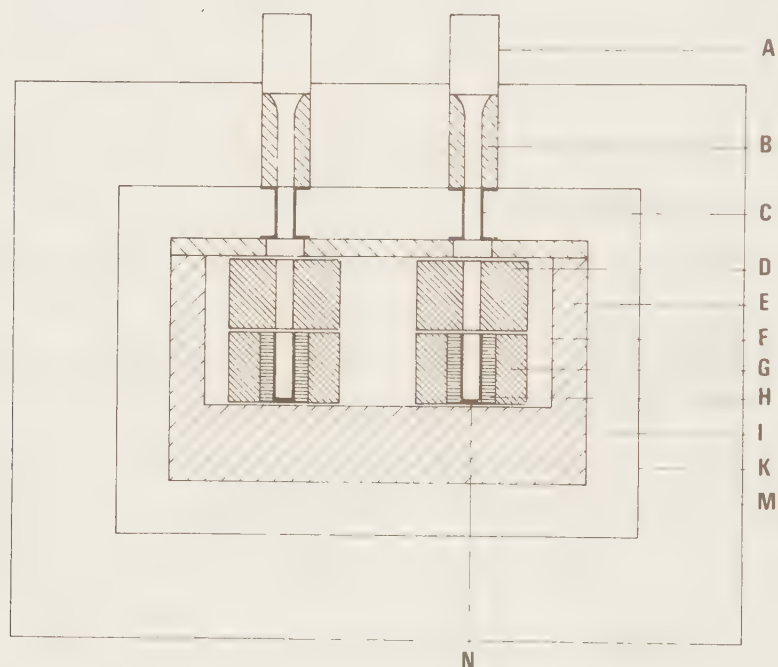


Fig. 1. Ampoule calorimeter

The positions A, B, C and D are successive resting positions to temperature equilibrate the ampoule. Thermoelectric element H, in contact with heat sink G, positioned in the main heat sink E. Air space F and I. Calorimeter jacket K, in a water thermostat M; ampoule holder N. Figure from reference (9).

A calibration constant, ϵ , was obtained electrically for both calorimeters. For the best results the heat liberated during calibration should closely follow that of a reaction to be measured. The position of the electrical heater in the flow vessel described in paper I was not found to be very important,

but the calibration constant varies with flow rate, paper I. In an ampoule the reaction volume can be accurately measured, however in a flow vessel this is not easy to obtain. It is usual to measure the volume of the vessel by filling the channels with water and weighing, but when in situ the apparent flow volume can vary due to the accumulation of heat between the equilibration coils and the measuring vessel. Consequently a known steady state reaction would be more suitable for determining the calibration constant. The hydrolysis of acetylcholine by choline esterase in Hepes buffer has been used for calibration (10).

2.3. Calorimetric vessels

The closed steel ampoule previously described was used to obtain microbial heat production for the identification of several lactic acid bacteria, paper V and for glycolytic activity, Fig. 6. For studies on catabolism by cell suspensions, papers IV and IX, a coiled gold tube positioned between two copper plates was used, Fig. 2A. For growth experiments, papers II, VI, VII and VIII the vessel used was constructed from a Teflon coil embedded in Woods metal, Fig. 2B. Teflon tubing allows the easy passage of gas

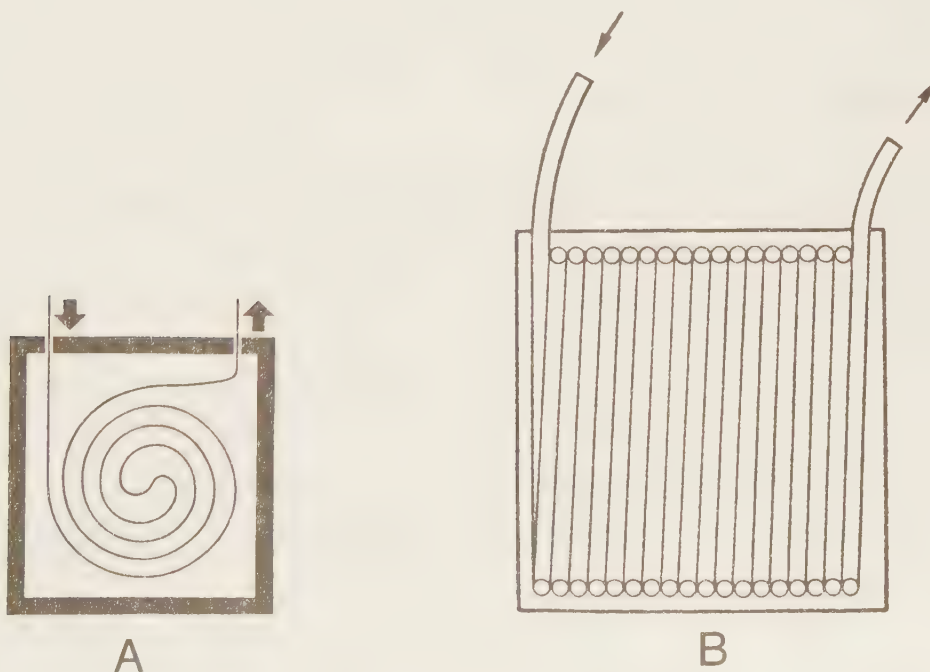


Fig. 2. A. Flow-through vessel constructed from a coiled gold tube, LKB-Produkter, Bromma, Sweden. B. Teflon tube embedded in Woods metal, I. Wadsö, reference (10).

bubbles formed in the flowing culture or transported from outside the calorimeter. The effect of oxygen on heat production during growth, paper III was examined in both the steel ampoule and the gold flow-through vessel.

2.4. Oxygen and pH control

For all calorimetric studies of microbial systems the adequate availability of oxygen or the establishment of anaerobic conditions must be known. The lactic acid bacteria studied were micro-aerophilic and their oxygen requirements could be satisfied by stirring or aerating the growing culture or suspension outside the calorimeter and transporting it at 40 ml per hour through Teflon tubing to the calorimetric vessel. Anaerobic conditions were obtained by enclosing cultures in steel ampoules under an atmosphere of nitrogen. Anaerobic conditions could be established in a flow calorimeter by stopping the flow once the vessel was full, the bacteria then utilized the oxygen in solution causing an anaerobic environment. These techniques were applied in papers IV and IX.

The production of acid by cultures in media of low buffering capacity such as the complex medium used in paper VII or the milk medium in paper VIII will cause a significant reduction in pH during growth. The decrease in pH can reduce the growth rate and the total cell yield complicating the interpretation of heat production where one variable is being investigated. The results in papers VII and VIII were obtained by pumping the growing culture through the calorimeter from a 1 l LKB Biotec fermenter maintained at 37 °C. The pH was controlled by the addition of a sodium hydroxide solution, automatically metered in response to the pH measured by a Radiometer pH meter. Results obtained with an ampoule calorimeter, paper V, were in a completely defined medium containing β -disodium glycerophosphate to increase the buffer capacity.

3. NON-GROWING CELLS IN PURE CULTURE

While anabolism cannot be examined separately from catabolism, the rate and regulation of catabolism can be studied by harvesting cells and suspending them in a suitable buffer. The energy made available by catabolism under these conditions becomes uncoupled from the synthetic reactions of the cell. Without an energy supplying substrate the cell will survive for a limited period by utilizing reserve materials previously accumulated during the stationary growth phase. This endogenous metabolism yields a measurable quantity of heat (5,11).

With a substrate present such as glucose, both non-growing and growing cells utilize part of the energy conserved by ATP formation to establish a membrane potential and a resulting pH difference by extrusion of protons (H^+) from the cell cytoplasm. This potential energy can be coupled to the active transport of many compounds across the cell wall.

The coupling of the energy made available by catabolism to the active transport is summarized by the chemiosmotic hypothesis (12), and has been extensively reviewed (13,14). An electrochemical potential difference is established by extrusion of H^+ out of the cell which also establishes a pH gradient, i.e.

$$\Delta p = \Delta \psi - Z \Delta pH$$

Where Δp , the protonmotive force (the difference in electrochemical activity for H^+), tending to drive H^+ in the opposite direction to their extrusion; $\Delta \psi$, the membrane potential in millivolts and $Z \Delta pH$ is the transmembrane pH gradient where $Z = 59$ at $25^\circ C$ is the factor converting pH to millivolts.

Hydrolysis of catabolically formed ATP by ATP-ases of the cell wall yields the energy to establish the proton-motive force to do this work (14). Conversely, a flux of H^+ into the cell can cause ATP generation (15). This is shown in Fig. 3.

Active transport can be uncoupled when the protonmotive force is collapsed or when the energy source providing the ATP is removed.

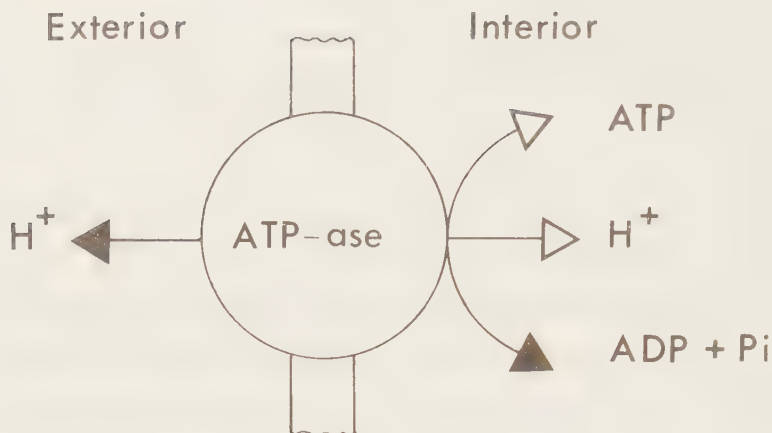


Fig. 3. Efflux of H^+ from the cell coupled to the hydrolysis of ATP and the influx of H^+ coupled to ATP formation, both reactions mediated by ATP-ase. Fig. from reference 15.

An uncoupling agent which is believed to solely collapse the membrane potential component due to protons and the pH gradient is 2,4-Dinitrophenol (DNP). Although glucose is taken up by group translocation, and not active transport, it is found that its rate of catabolism is stimulated by uncoupling agents (15) and papers IV and IX.

It has been postulated that the increased H^+ flux in the presence of DNP stimulates ATP-ase activity and glucose catabolism increases in response to the reduced level of ATP within the cell (15). For Streptococci there is no evidence of catabolic activity being regulated by the demand for available energy mediated through the ATP pool, either in washed suspensions, or during growth (16).

In papers IV and IX investigations on the action of DNP in stimulating glucose catabolism by Streptococci were made. In paper IV it was established that both the aerobic and anaerobic catabolism could be measured by flow calorimetry and there were similarities between the maximum stimulation of catabolism by DNP and the

transitory elevated rate obtained immediately on the addition of glucose. This study was enlarged in paper IX to determine if the pH change in the cell on glucose catabolism or by the action of DNP as a proton conductor was involved in the observed rate of catabolism.

Comparison between suspensions of S. faecalis and S. agalactiae showed increased glucose catabolism depending upon the concentration of DNP and the age of the cells when harvested. S. faecalis required about 100 μ M DNP for maximum glucose catabolism, while S. agalactiae required about 250 μ M DNP. Untreated cells of S. agalactiae gave an initial rapid rate of glycolysis on the addition of glucose and this was maintained for about 8 min and then a lower steady state was established. The lower rate appeared related to the cell establishing a different pH. A change in rate of glucose catabolism by S. faecalis, was not observed, however in this bacteria the change has been reported as occurring rapidly, within a few seconds of glucose addition (W.W. Forrest, personal communication).

The maximum stimulation with DNP was similar to the initial elevated transitory rate observed on the addition of glucose to untreated suspensions. Anaerobically without DNP, both strains maintained a constant glycolytic rate essentially independent of the buffer pH. With DNP present the glycolytic rate responded to the buffer pH and this was attributed to the action of DNP in equilibrating the pH of the cell cytoplasm with that of the buffer.

The presence of oxygen did not change the rate of glucose catabolism by DNP treated or untreated suspensions, however increased heat production was observed resulting from the oxidation of metabolically formed pyruvate. In the absence of DNP the aerobic heat production by S. faecalis, but not S. agalactiae, was sensitive to buffer pH.

It was concluded that increased glucose catabolism in the presence of DNP appeared to be determined by the pH within the cell which changed in response to DNP acting as a proton conductor.

4. GROWING CELLS IN PURE CULTURE

Microbial growth is made possible by energy coupling between the catabolic and anabolic reactions of the cell. The study of growth can be simplified when the substrate being catabolized only provides the energy for growth and none or very little of its carbon is incorporated into the cell. This situation occurs with lactic acid bacteria when growing in a nutritionally rich environment and the energy provided by catabolism is used to polymerize the compounds in the growth medium into cellular structure and functional compounds (17).

Under anaerobic conditions power-time curves produced by lactic acid bacteria growing in nutritionally complex medium with limiting amounts of a carbohydrate energy source have consistently shown simple profiles (4,18), as have Z. mobilis (19) and E. coli (20). These results reflect balanced growth where one rate constant is found for heat production, growth rate, degradation of substrate and formation of products. It has been proposed and restated in paper VII that the observation of a single rate for exponential heat production by a growing culture is a sensitive measure of balanced growth.

Under anaerobic conditions the dry weight of cells produced by growth on a limiting amount of energy source is constant when expressed in terms of the amount of ATP generated (17). The yield coefficient Y_{ATP} , of about 10.5, is defined as the number of grams dry weight of cells produced for each mole of ATP generated by catabolism. This yield is thought to reflect the upper limit of energetic coupling between ATP formation from substrate catabolism and its use in anabolic reactions (16). Calculations on the ATP requirements for synthesis of cellular material from simple precursors in the medium gives 0.037 moles of ATP for each g. dry weight of cells or a Y_{ATP} of 27 (21). A large amount of ATP formed by catabolism is therefore used for other purposes such as maintenance, active transport or lost by hydrolysis.

While developing a method for the identification of bacteria of possible importance to the Dairy Industry, paper V, it was found

that the culturing history of the inoculum could also influence the shape of the power-time curve obtained. This led to a more detailed examination of the affect of the inoculum preparation and the presence of oxygen in determining the observed heat production. These observations are reported in paper VII.

A single exponential increase in heat production until all the glucose had been degraded, was obtained for S. lactis depending upon the method of inoculum preparation. A culture grown in milk medium with lactose as the energy source, and then passaged once in complex medium containing excess glucose, subsequently gave a simple power-time curve in complex medium containing a growth limiting concentration of glucose. In the absence of glucose essentially no growth occurred in the medium during the time that glucose was being utilized by the other culture. This result was interpreted as indicating that glucose was the only energy source utilized. The culture gave similar rate constants for heat production, cell yield and the rate of glucose degradation. This aerobic growth however gave a molar growth yield (MGY) of 54.6 g dry weight of cells for each mole of glucose degraded, compared with a MGY of about 21 which is consistently found for many similar bacteria under anaerobic conditions. This indicates a very high coupling efficiency assuming that 3 moles of ATP were produced for each mole of glucose degraded giving a Y_{ATP} of 18.2 compared with 10.5 for anaerobic conditions. However this high value is similar to those reported for aerobic growth of S. diacetylactis and S. cremoris of 20 (22).

Because of the numerous reports of a maximum coupling efficiency of 10.5 g dry weight of cells per mole of ATP other reasons for the high growth yield under aerobic conditions must be considered before accepting this value as being the result of an increased metabolic efficiency, rather than an increased ATP production. Streptococci are known to acquire a cytochrome-like system which could lead to an increased yield of ATP, however this only occurs in organisms grown in a medium to which haematin has been added (23,24). Another energy source could have supported growth concurrently with glucose catabolism, however it is unlikely that the rate of glucose degradation, heat production or cell yield

would be the same as found with this culture. The culture was able to grow on medium components other than glucose after it had been passaged ten times in the complex medium.

Further analysis of the data obtained from the power - time curves after adaption of S. lactis to growth in the complex medium has shown regulation in the rate of heat production and hence growth rate of the culture on the unknown compound degraded before glucose. This regulation was effected by glucose concentration in the medium. Fig. 4 summarizes these results where the initial rate of heat production was used to calculate doubling time and is expressed as a function of glucose concentration. No degradation of glucose could be measured at this stage of growth.

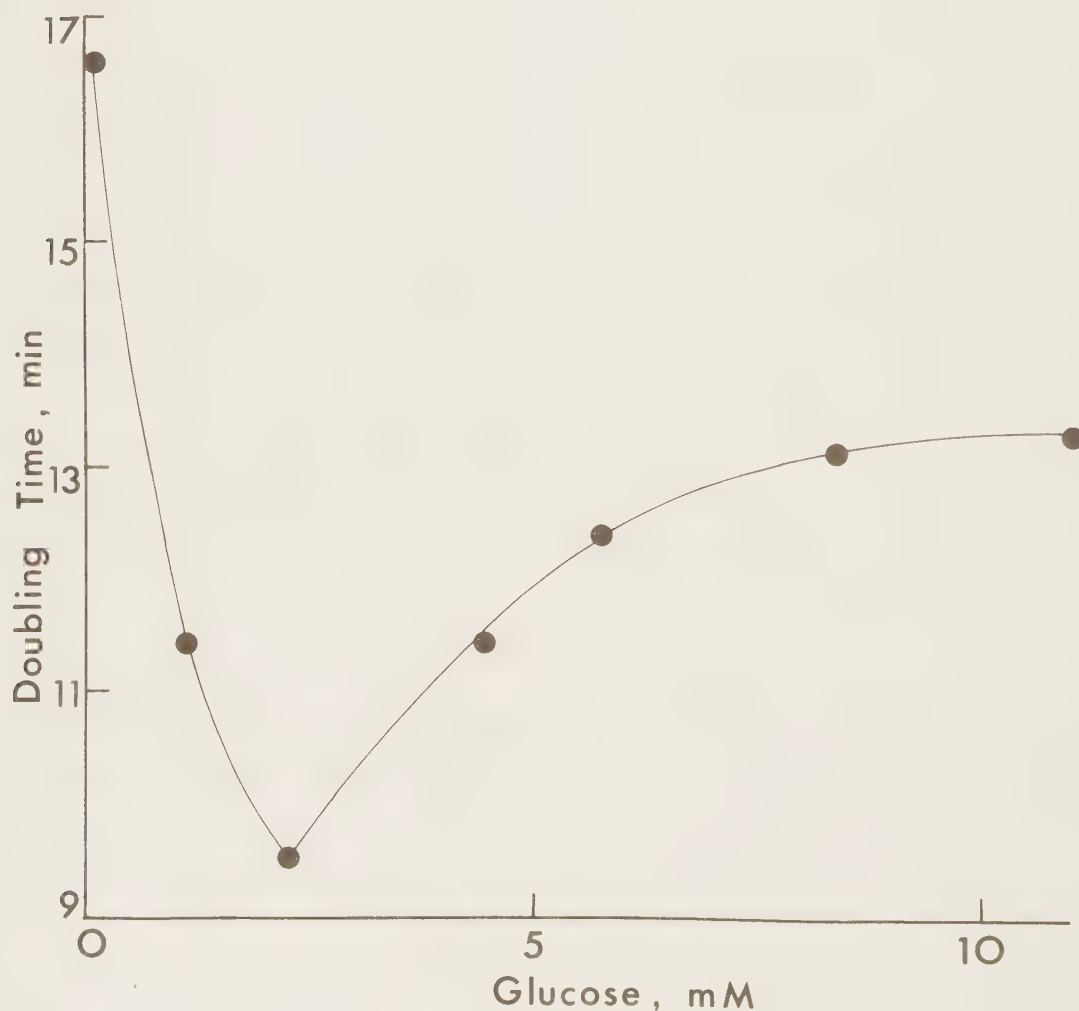


Fig. 4. Influence of glucose concentration on the initial growth rate of S. lactis in complex medium before measurable glucose degradation. P. Monk, unpublished results.

As glucose concentration increased, the doubling time of the culture decreased reaching a maximum rate at 2 mM glucose. The growth rate then slowly decreased to a constant value higher than that found in the absence of glucose. Although glucose degradation could not be observed during the period of its influence, the analytical method may not have been sufficiently sensitive to detect the small amount of glucose taken up by the culture to exert this affect. Glucose appears to exert catabolic stimulation of the initially preferred energy source while its own degradation is inhibited until the compound being degraded has reached a lower concentration in the medium. This complex interaction between two energy supplying substrates and cell growth may also have occurred where balanced growth was observed causing an increased efficiency in substrate utilization.

After adapting the culture to grow in complex medium with a reproducible metabolism the power-time curve became profiled and at no stage did balanced growth occur. It was shown that changes in the rate of heat production could, in general, be related to corresponding changes in either growth rate or glucose degradation. These changes were interpreted as corresponding to the use of other energy supplying compounds in the medium. Apparent molar growth yields for glucose could be calculated for different stages of growth and showed variation resulting from cell yield from other medium compounds. The overall MGY of 31.4 found after correction for cell yield on the complex medium without glucose was the same as found by Forrest and Walker (25) for S. faecalis at low glucose concentrations grown without anaerobic precautions.

Although aerobically grown cultures show varied rates of heat production and glucose catabolism, the glycolytic ability of the cells remains constant. In Fig. 5 is shown the heat production curve obtained for S. faecalis growing aerobically in a complex medium containing glucose.

During growth, samples were removed, centrifuged and suspended in phosphate buffer containing glucose. The optical density was measured as an index of growth and the heat produced during anaerobic catabolism determined in an ampoule calorimeter to

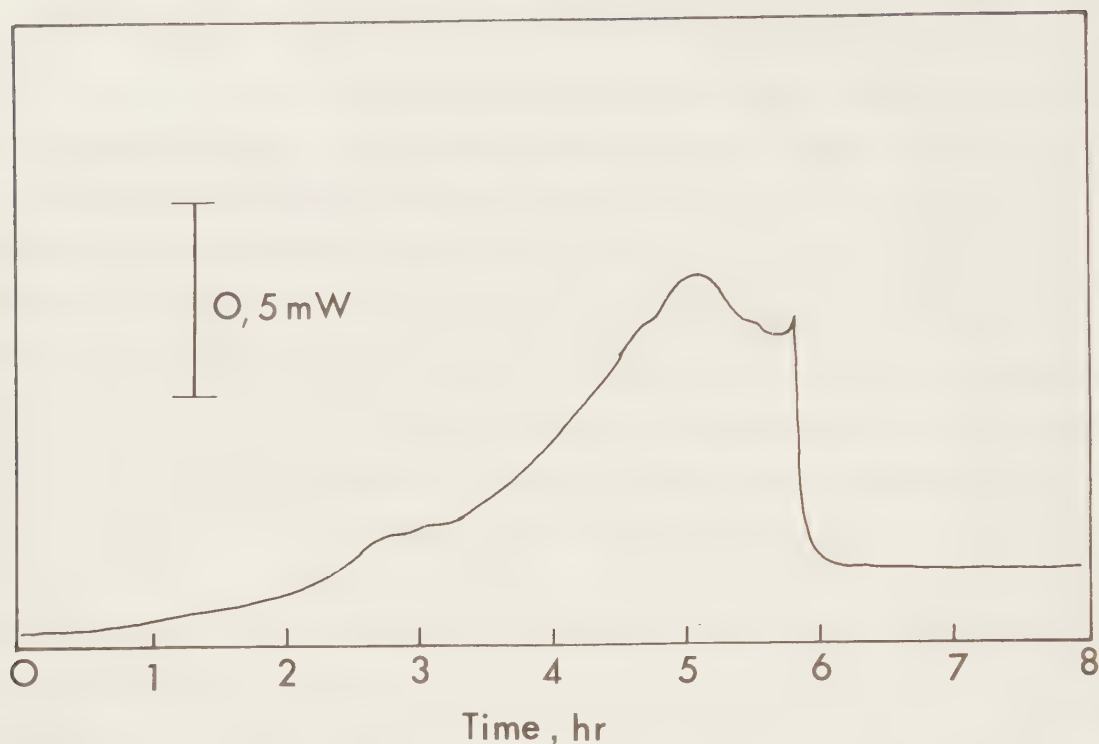


Fig. 5. Heat production by S. faecalis growing in complex medium containing glucose with pH maintained at 7. P. Monk, unpublished results.

measure glycolytic activity. The results are shown in Fig. 6. The growth rate of the culture and the increase in glycolytic activity had similar rate constants. Although glucose catabolism may be regulated during growth the ability of the cells to degrade glucose remains constant for a unit mass of cells.

The results in paper VII indicated the influence of medium composition, and the method of inoculum preparation on the characteristics of the profile obtained. While for the purpose of microbial identification (cf. paper V) these parameters would require careful control, the power-time curves are inherently useful in observing energetic regulation and coupling.

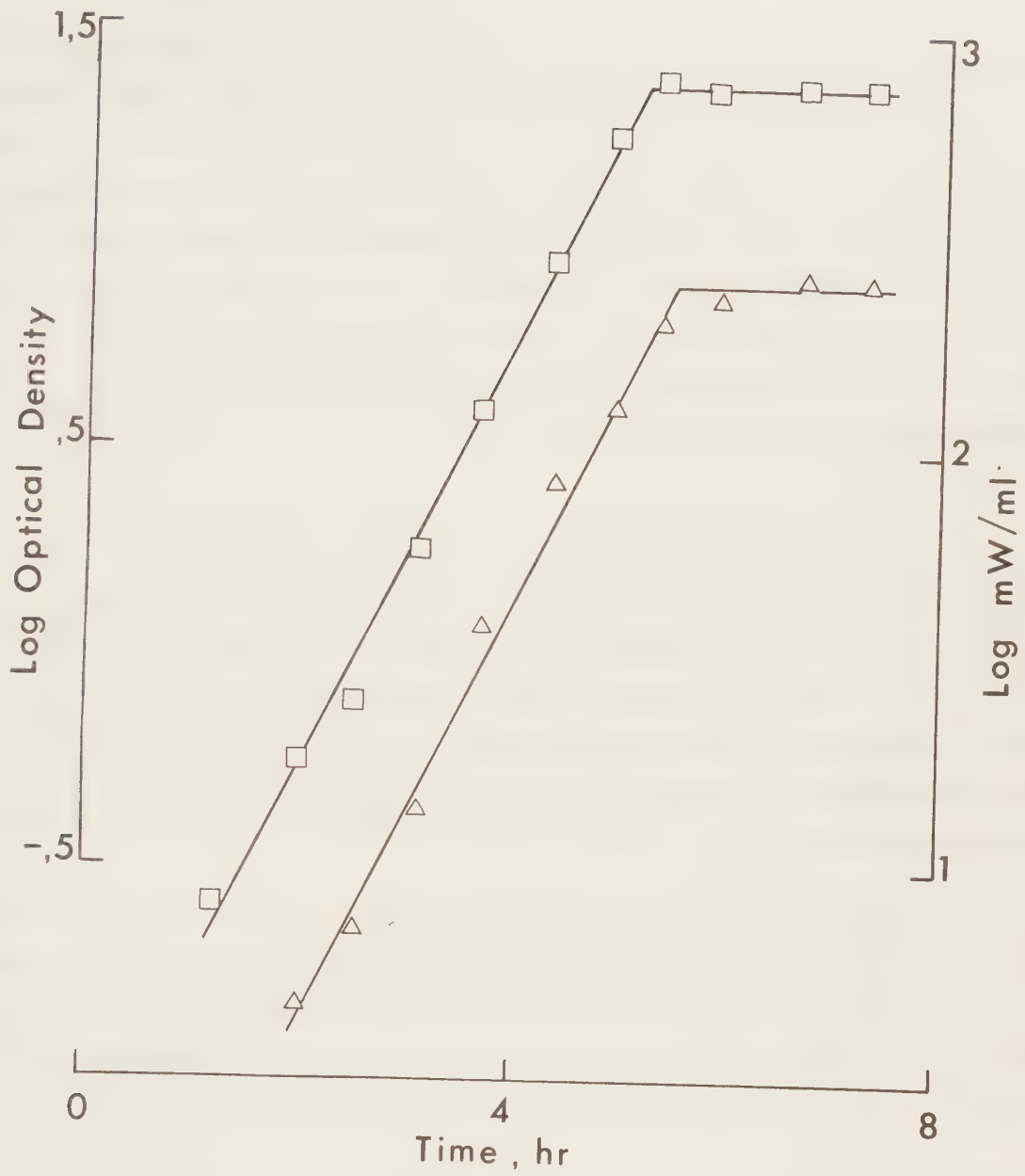


Fig. 6. Semi-logarithmic plot of growth rate and glycolytic activity during growth of *S. faecalis* shown in Fig. 5.
P. Monk, unpublished results.

5. GROWING CELLS IN MIXED CULTURE

In natural environments mixed cultures are commonly found and are usually composed of many different organisms which have developed a symbiotic relationship. The optimum operation of such systems is dependent upon a delicate balance in the population. Where some enforced variable occurs the regulation of the system may get out of control causing economic or environmental problems. Fermentations as different as sewage treatment, ruminant fermentations, methane production from waste and preparation of food products are typical examples.

Monitoring of mixed populations is a difficult procedure both routinely and when examining the affect of controlled changes in variables. Heat production has been successfully used to investigate rumen fermentations (26), soil (27) and sewage treatment (28). The complex microbial populations, accompanied by the simple power-time curves usually obtained, have discouraged attempts to characterize interactions between strains and contributions to the heat production by individual strains (29).

Of importance to the Dairy Industry is the use of mixed cultures of Streptococcus thermophilus and Lactobacillus bulgaricus for the production of fermented milk products. This fermentation was selected for examination by calorimetry and the results presented in paper VIII.

Although the power-time curves of both strains in milk medium were simple, the pure cultures could be recognized by their difference in growth rate and time of fermentation. Growth of the mixed culture also showed simple power-time curves, but the effect of inoculum properties on the course of the fermentation could be analysed. At a constant ratio of L. bulgaricus to S. thermophilus of 3.8:1 the amount of inoculum used only affected the lag time while growth rate, maximum power output and total heat production remained constant. The mixture behaved essentially as a single culture in relation to increased lagtime with decreasing amounts of inoculum. The stimulation in growth of S. thermophilus by L. bulgaricus also remained constant.

With different proportions of the two strains in a fixed volume of inoculum, the growth rate of the culture could be increased by decreasing the numbers of L. bulgaricus without significantly reducing the stimulated growth of S. thermophilus.

When an originally mixed culture was repeatedly sub-cultured the power-time curve showed an increasing dominance of S. thermophilus in the mixture, but a reduction in its growth resulting from a reduced supply of growth factors from L. bulgaricus. The properties of the mixed culture could not be maintained by repeated subculture. Other mixed strains, however have shown constant properties (30).

The stimulation of S. thermophilus by growth factors formed by L. bulgaricus was shown by the addition of cell free filtrates to the growth medium. However, both cultures seemed to improve the growth of each other. Using the increase in rate of heat production as an indicator of balanced growth, it was found that a single exponential described the growth of the mixture, but three different growth rates were found for both of the single cultures.

It could be concluded that power-time curves of mixed cultures, although relatively simple in form, were useful in evaluating the effect of different variables on the overall metabolism. Selection of different strains for incorporation into mixed cultures could be examined for compatibility and stability.

The use of calorimetry in evaluating mixed cultures was applied to the compatibility of strains and reported in paper VI. A strain of S. cremoris was unable to grow with S. lactis which dominated the fermentation even when the ratio of the two strains in the inoculum was 1:10. S. lactis had a much shorter lag time than S. cremoris, however when the lag time of the mixed culture was increased by decreasing S. lactis in the inoculum, S. cremoris was still unable to grow. It appeared that factors other than relative growth rates influenced the dominance by S. lactis. A characteristic shoulder on the power-time curve recorded for S. lactis remained constant in response and size and was used as a marker.

6. MICROBIAL CHARACTERIZATION USING HEAT PRODUCTION

A microorganism is expected to show balanced growth under conditions of adequate nutrition and when catabolizing one energy supplying substrate. Power-time curves of balanced growth are simple and therefore are of limited use for identifying growing cultures. A profiled growth pattern can be a sensitive indicator of deviations from optimum growth conditions.

Among those factors which can influence the shape of the power-time curve obtained are medium composition, the physical environment and the previous culturing and storage conditions of the inoculum used. Unless these factors are carefully controlled a reproducible result might not be obtained.

6.1. The effect of oxygen

When lactic acid bacteria are grown aerobically in complex medium their patterns of heat production can become profiled, as shown in paper III. It was determined that the presence of oxygen caused the complexity, because when grown in closed steel ampoules or when the flow was stopped and the culture allowed to grow in the calorimetric vessel, the power-time curve became simple and bell shaped. In Fig. 7 these results are shown for a culture of S. faecalis var. liquefaciens studied in paper III.

The bacteria were able to consume the oxygen present under the static conditions creating an anaerobic environment.

6.2. The effect of medium composition

Aerobically grown cultures can be examined by flow calorimetry. A suitable arrangement of apparatus for monitoring the growth of E. coli is shown in paper II. When grown aerobically in a commercially available complex medium the record of heat production was profiled and essentially reproducible. Intervals of low heat production between the main thermal events are believed to indicate the induction of enzyme systems for catabolism of other medium components or products of the initial fermentation.

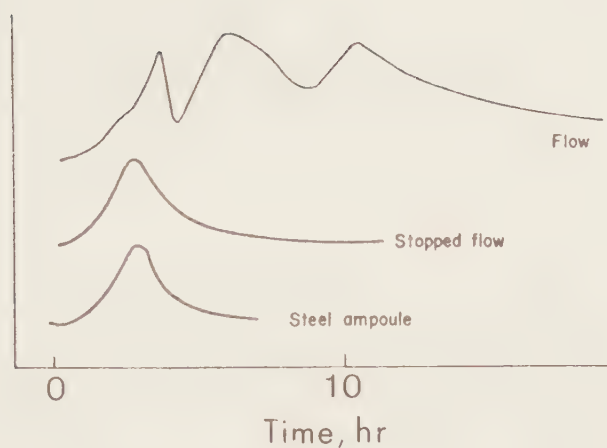


Fig. 7. Growth of S. faecalis var. liquefaciens in a complex medium with and without oxygen. From paper III.

The complexity of the power-time curve and the total heat production depended upon the amount of yeast extract added to the medium, as shown in Fig. 8.

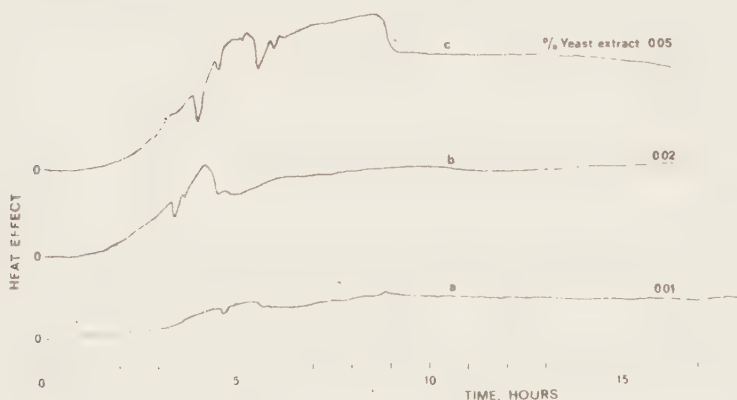


Fig. 8. Power-time curves for the growth of E. coli on 0.25 % glucose solution, salt medium and different concentrations of yeast extract. From paper II.

In a medium containing yeast extract and salts, but no glucose only the initial part of the original profile was observed.

Peaks occurring after an induction period were dependent upon the presence of glucose. Small variations in the concentration of either glucose or yeast extract would therefore be expected to give different results for the same culture.

6.3. The effect of inoculum size

The amount of inoculum and its viability used to initiate growth will determine the lag time of a culture before the onset of exponential growth. While a power-time curve might retain its characteristic events independent of the inoculum size, the time at which they occur will be displaced. Inoculum size and lag time are generally related as shown in Fig. 9, for the growth of S. faecalis in a complex medium.

The lag time increases with decreasing amounts of inoculum, therefore to obtain reproducible displacement of thermal events, the viable inoculum size should be carefully adjusted. Exponential growth remains constant over a wide range of inoculum size and independent of lag time, however at low levels of inoculum the subsequent growth rate of a culture can change. This will be reflected in the rate of heat production and will influence the shape of the power-time curve. The growth rate at different levels of inoculation is shown in Fig. 10.

6.4. Effect of freezing inoculum

In the development of methods for the identification of lactic acid bacteria, paper V, the frozen stock cultures had to be subcultured several times, depending on strain, before a reproducible power-time curve could be obtained. This observation is important in relation to altered metabolic properties brought about by freezing, when using frozen starter cultures in the Dairy Industry.

The effect of low temperature storage on starter cultures has been investigated in terms of cell wall changes, enzyme levels, ability to accumulate peptides and growth performance (31). However, metabolic changes are not easily recognized by these methods. Calorimetry has been successfully used to determine the viability of yeast cultures after storage in liquid nitrogen (32).

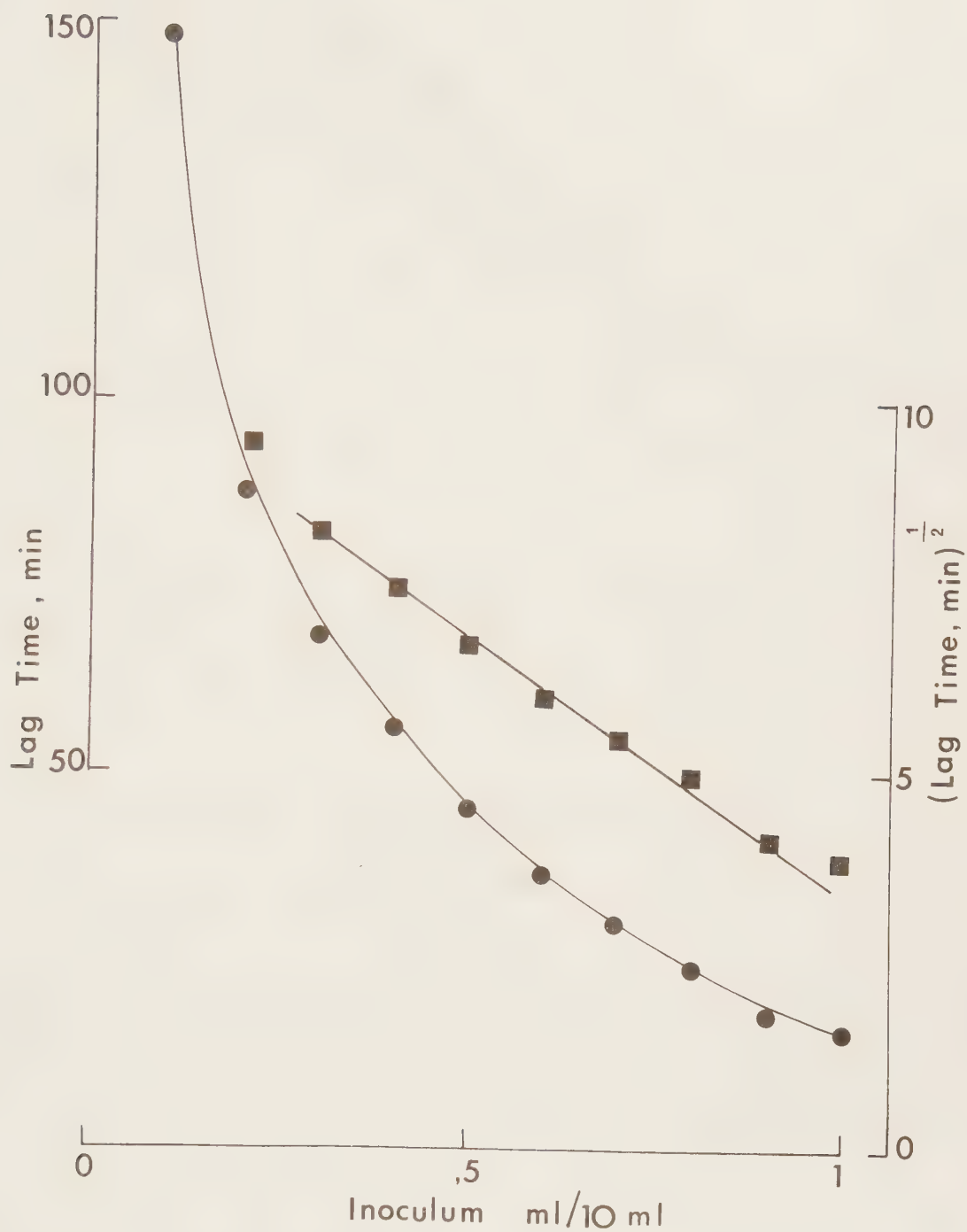


Fig. 9. Relationship between the amount of inoculum and the lag time for *S. faecalis* growing in a complex medium.
P. Monk, unpublished results.

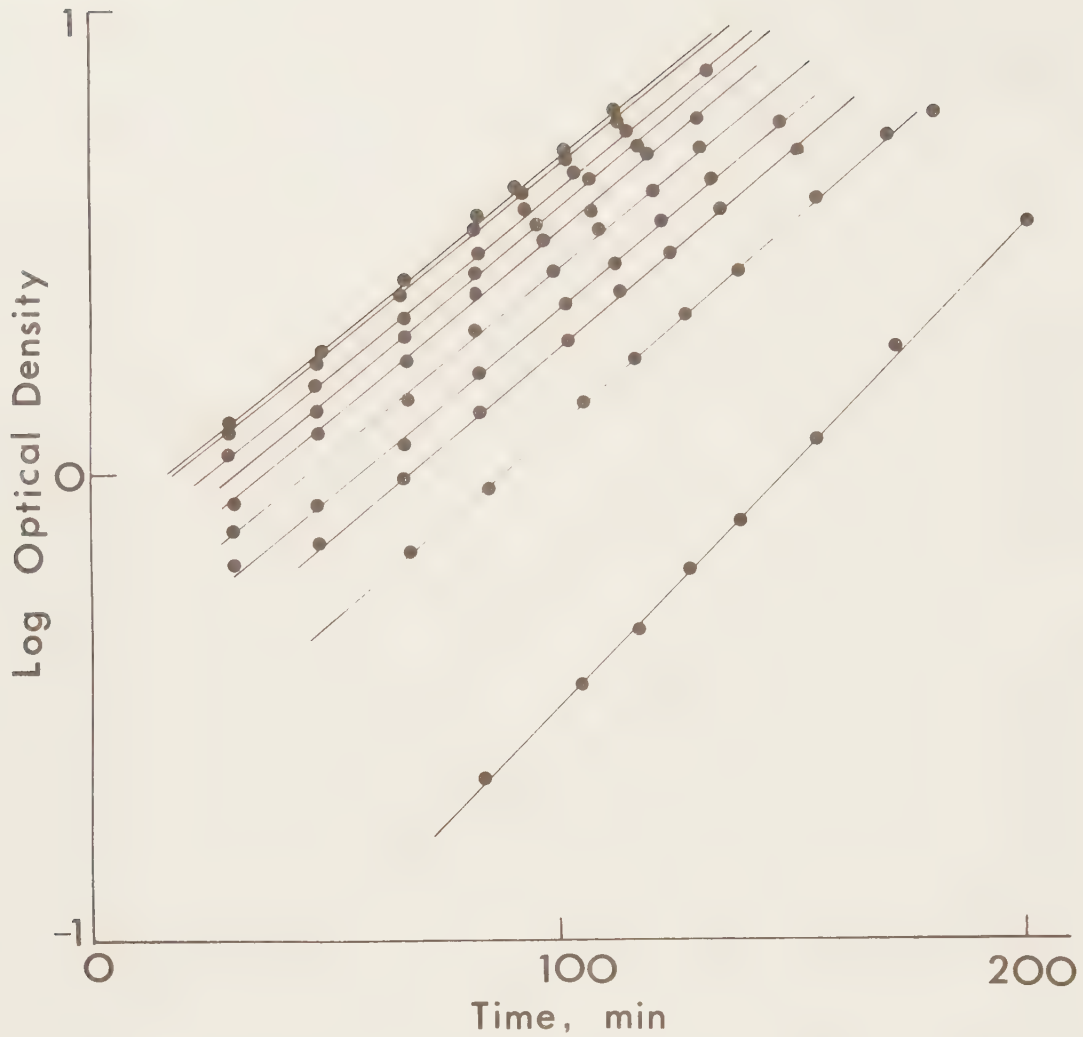


Fig. 10. Relationship between amount of inoculum and growth rate for S. faecalis growing in a complex medium. The amount of inoculum used decreased in increments of 1 % from 10 to 1 %; top to bottom. P. Monk, unpublished results.

As reported in paper VI, the heat production by S. lactis grown in a defined medium containing lactose changed, depending upon the storage time at -22°C . The ability to utilize one medium component became impaired with time, Fig. 11. With this strain only one sub-culture was required for its metabolic ability to be restored. Other cultures required several sub-culturing steps before giving the original result.

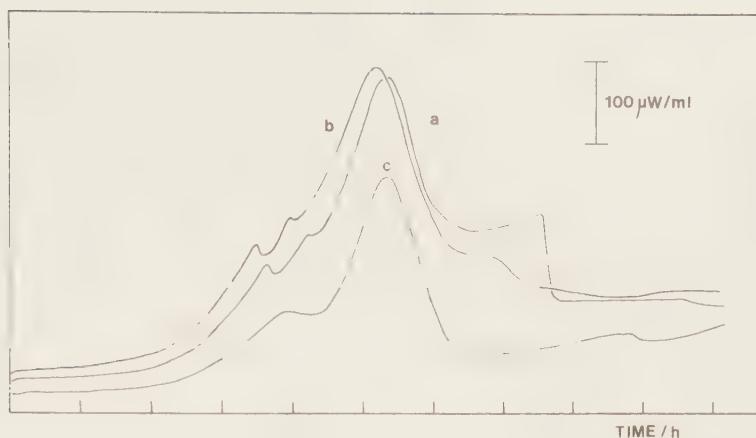


Fig. 11. Affect of storage time on the fermentation properties of frozen inoculum of *S. lactis*. Curve (a) shows growth using unfrozen inoculum, (b) inoculum frozen for 3 days and (c) for 17 days at -22°C . From paper VI.

6.5. Identification of lactic acid bacteria

The aim in using heat production for the identification of bacteria should be to obtain a reproducible expression of an organisms metabolic capability, while emphasizing differences between strains. As the heat production mainly reflects the catabolism of substrates, a complex power-time curve can be obtained by modifying the type and amount of substrates in the medium while providing adequate amounts of other growth factors. This approach was applied to the identification of several lactic acid bacteria. The results are reported in paper V and the heat production from some mesophilic strains shown in Fig. 12.

Growth in complex medium of both mesophilic and thermophilic bacteria gave results which were not significantly different to allow their differentiation. A completely defined medium containing a mixture of carbohydrates added in growth limiting amounts gave power-time curves which were characteristic of individual strains.

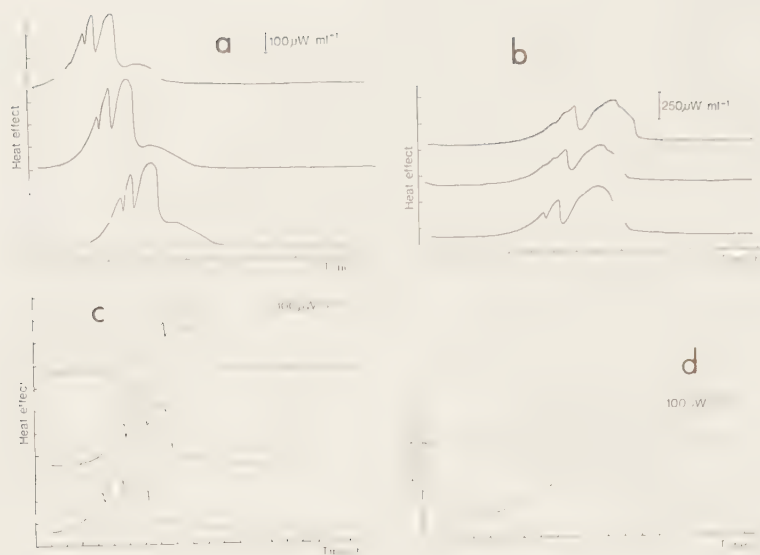


Fig. 12. Growth of some mesophilic lactic acid bacteria in defined medium at 37 °C. (a) S. cremosis; (b) S. diacetylactis; (c) S. lactis; (d) Leuc. cremoris. Three replicates are shown for each strain and displaced on the response axis for clarity. From paper V.

The method was considered feasible for recognizing a limited number of commercial strains and for monitoring contamination or changes in their fermentation properties.

6.6. Compatibility of strains in mixed culture

Strains of bacteria selected for incorporation into mixtures must be compatible to contribute the desirable properties observed in pure culture. Many strains do not grow together due to differences in growth rate, production of inhibitors or might not demonstrate stability on repeated subculture.

In paper VIII the growth of S. thermophilus and L. bulgaricus, both in pure culture and as mixtures could be described from their heat production during growth. Calorimetry can also be used

to select strains for incorporation into mixtures if they can be recognized from their power - time curves.

In paper VI a mixture of S. lactis and S. cremoris was examined for compatible growth. From growth in a defined medium containing lactose, the results obtained could be used to recognize the dominance of S. lactis while S. cremoris was unable to grow in the mixture even at inoculation levels ten-fold over that of S. lactis. Their heat production during growth in pure and mixed culture are shown in Fig. 13.

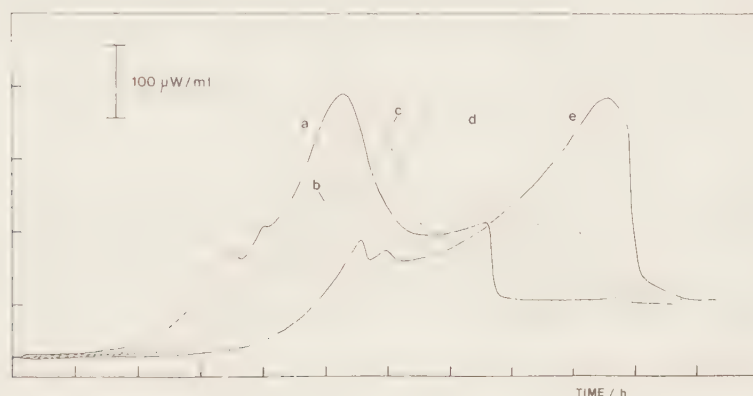


Fig. 13. Power - time curves of S. lactis and S. cremoris grown in mixed culture at 37 °C. Medium inoculated at the 1 % level with an 8 h culture of S. lactis (a) and S. cremoris (e). Mixed inoculum containing ratios of S. lactis to S. cremoris of 1:1, 1:2.5 and 1:10 generated the curves (b), (c) and (d) respectively. From paper VI.

7. SOME CONCLUSIONS

1. The calorimeters available have sufficient sensitivity for the general study of microbial metabolism.
2. Aerobic conditions can be established in flow calorimeters for micro-aerophilic cultures where sufficient dissolved oxygen is transported with the liquid flow. Aerobic cultures require

calorimeter vessels capable of handling air pumped with the liquid. Anaerobic conditions can be achieved in flow vessels by those cultures which utilize the dissolved oxygen when the flow is stopped. However further attention should be given to designs to allow flow systems to be operated under strictly anaerobic conditions.

3. Calibration of flow vessels is subject to error due to the difficulty in accurately determining the effective volume at different flow rates. Baseline shifts during the course of a measurement can occur due to changes in viscous heating.
4. Sealed ampoule calorimeters can be made anaerobic and are easily calibrated, however they are limiting in regards to mixing, addition of reagents or where the reaction produces a gas.
5. Any particular micro-organism may exhibit many different power - time curves depending upon the culturing history of the inoculum used, the composition of the growth medium and the physical environment in which it is grown. A result may be modified by the selective alteration of any of these variables. However, growth conditions can be controlled to obtain power - time curves for the identification of bacteria. The variation in the power - time curve of a culture offers an advantage in studying its metabolism.
6. Simple mixtures of cultures can be examined by calorimetry to yield information about their compatibility and the affect of varying the growth conditions.

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A Flow Micro Reaction Calorimeter

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A Flow Micro Reaction Calorimeter

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A flow micro reaction calorimeter has been designed and tested. The construction is a twin heat conduction calorimeter. Heat evolved in a flow reaction cell is conducted to a surrounding heat sink through a semiconductor thermopile. Electrical calibration experiments indicate the precision at steady state conditions to be 0.1 % if the heat effect is $>100 \mu\text{cal/sec}$. Heat effect sensitivity is approximately $0.1 \mu\text{cal/sec}$. Test experiments involving mixing efficiency in a flow cell and heat of dilution of urea and sucrose solutions are reported and discussed. A comparison is made between the properties for the present flow calorimeter and those for the batch calorimeter on which design it is based.

Flow reaction calorimetry has several advantages over a batch calorimetric method. The operation at a calorimetric experiment can be made exceedingly simple and equilibration time prior to the experiment can be omitted. Mixing of reactants can be achieved without the presence of a gaseous phase which is of great importance when experiments are performed with volatile liquids and in micro-calorimetric experiments where very small condensation-evaporation effects may affect the result. Surface adsorption effects which may cause serious systematic errors in micro calorimetry can be neglected if a steady liquid flow is allowed to continue until possible wall reactions have occurred.

Recently a precise micro reaction calorimeter based on the heat conduction principle was reported.¹ The reactants were contained in a mixing vessel of two compartments and the reaction was started by rotation of the calorimeter block. In this paper is described the design of a liquid flow calorimeter which is based on the same principle as the earlier reported batch calorimeter.

Several liquid flow calorimeters have been reported. They were designed primarily for studies of reaction rates² or enthalpy titration (see, *e.g.*, Refs. 3, 4). Flow reaction calorimeters have also been reported.^{5,6} The calorimeter

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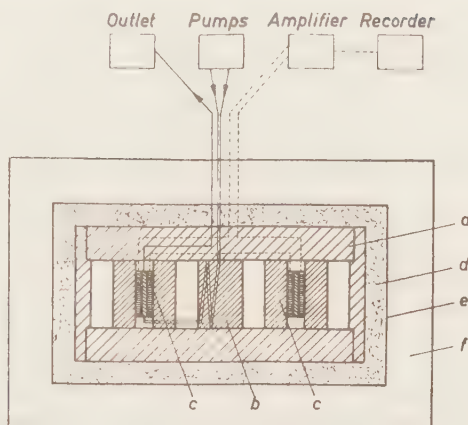


Fig. 1. Principal arrangement of the calorimeter.

described by Stoesser and Gill⁶ is, similar to the present construction, a micro calorimeter designed for precise work.

GENERAL DESCRIPTION OF THE APPARATUS

The arrangement of the apparatus is shown in Fig. 1. A metal block heat sink, *a*, contains a centrally located heat exchange unit, *b*, surrounded by calorimetric units, *c*, in a twin arrangement. The calorimetric units consist of flow reaction cells surrounded by surface thermopiles which are in contact with primary heat sinks. The heat sink is thermally insulated and immersed in a thermostated water bath. The calorimetric liquids are pumped through the heat exchange unit to one of the reaction cells and from there to a receiver outside the calorimetric system.

The voltage signal recorded at an experiment is the differential signal from the two thermopiles and external thermal disturbances are therefore largely expected to cancel. Liquid flows only through one cell during an experiment and a correction is applied for zero effects arising from liquid flow.

CALORIMETRIC PRINCIPLE

Two calorimetric liquids are brought together in a mixing zone of the flow cell. A constant flow rate will give rise to a constant heat effect in the calorimetric cell. If this heat effect is allowed to continue long enough steady state conditions will be established, *i.e.* heat generated in the cell will per unit time be equal to the heat transported out from the cell. Utilization of steady state conditions is the most accurate method of operating the present calorimeter and is the principle which will be discussed here.

A major part of the heat effect generated in the cell (W) will at steady state be transported from the cell by heat conduction through the surrounding thermopile (W_t), and to a minor extent will be lost through the air gap between

parts of the flow cell surface not in contact with the thermopile. A small fraction of the total heat effect generated will also leave the cell by the liquid flow.

It may be expected that for a given calorimeter and for stated values for flow rate, reaction rate and physical properties of the calorimetric liquid the fraction W_t/W will be constant

$$W = \alpha \cdot W_t \quad (1)$$

The transversal temperature gradient at various parts of the thermopile is expected to be proportional both to the heat flow and to the voltage generated. Integration over the total thermopile area will lead to the expression

$$W_t = \beta \cdot V \quad (2)$$

where β is a constant and V is the thermopile voltage.

Combination of (1) and (2) will lead to

$$W = \alpha \cdot \beta V = \varepsilon \cdot V \quad (3)$$

At an experiment a small fraction of the heat effect generated in the cell is a consequence of the flow of liquid through the cell such as heat of friction etc. The contribution to the thermopile signal from this latter heat effect may be determined at separate zero experiments. If this thermopile value is taken as the reference point for the thermopile voltage at the main experiment then

$$W_p = \varepsilon \cdot V_p \quad (4)$$

where W_p is the heat effect connected with the process occurring in the flow cell and V_p is the displacement of the thermopile voltage value from the determined baseline. The constant ε is determined by electrical calibration.

CONSTRUCTION DETAILS

Main heat sink. The main heat sink, *a*, Fig. 1, is similar to that which was described earlier for the batch calorimeter.¹ It is an aluminium cylinder of two parts, diameter 150 mm and length 200 mm. The cylinder has a central 75 mm bore and is fitted with 10 mm end walls. In the bore is contained the heat exchange unit, *b*, and the calorimetric units, *c*.

The aluminium cylinder is covered by a 20 mm layer of polystyrene foam, *d*, and the construction is surrounded by a cylindrical stainless steel jacket, *e*, with a lid fitted with an O-ring gasket. The jacket rests horizontally on a support in a thermostated water bath, *f*, which has a temperature fluctuation of about 0.005°C. Electrical leads and tubings for the calorimetric liquid are taken from the jacket through the bath by a 10 mm steel tube.

Heat exchange unit. The heat exchange unit, *b*, consists of 0.6 mm (*i.d.*) gold tubes which are bedded by tin in a 75 mm brass bolt. In the present construction the gold tubes are about 500 mm long. An established thermopile signal was not affected by a 25°C change in temperature of water pumped into the calorimeter at a rate of 0.17 ml/min.

Calorimetric units. The calorimetric units, *c*, have a similar sandwich type construction as was used in the batch calorimeter.¹ The flow cell which has the external shape of a squared plate is in good thermal contact with the thermocouple plates positioned on either side. These consist of commercially available thermoelectric coolers (Thermoelectric Modulus 3951-1, Cambion, Cambridge, Mass.). Electrical leads from the two thermocouple plates are connected in series. The external surfaces of the thermocouple

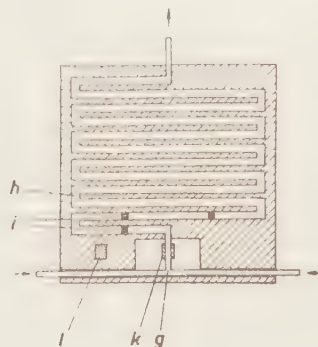


Fig. 2. Section through the flow cell.

plates are in thermal contact with aluminium bolts (75 mm in diameter, 25 mm thickness) acting as primary heat sinks. The calorimetric unit is joined together by three 6 mm screwbolts.

Flow cells. During the course of this work several flow cells of different design were tested. Part of the experience gained will be referred to in a later section of this paper. Here will be described that version which was finally adopted for fast reactions and which was used for the test experiments reported.

A section through the cell is shown in Fig. 2. The cell consists of a flat squared metal plate housing a T-piece, *g*, which leads to a channel system, *h*, milled out from the surface of the plate. The open channel is covered by a lid (a 0.6 mm plate) and the construction is sealed with epoxy resin (Araldite AY103). Between the plate and its channel system and the lid there is positioned a 0.05 mm 24 K gold foil acting as a gasket. The construction material for the plate and the lid is pure silver but all surfaces in contact with the calorimetric liquid are gold plated.

The two incoming streams of liquid are brought together in a gold T-piece positioned in a hole in the cell plate. A gold tube carries the liquid flow to the channel, where, in the mixing zone, three constrictions, *i*, are arranged to ensure complete mixing of the reactants. Each constriction is a plastic plug (Delrin) punctured to form a small hole. The inner dimension of the gold tubes forming the T-piece is 0.6 mm. The channel has a squared section, 1.5×1.5 mm, and the constriction holes are about 0.2 mm.

A 30 ohm calibration heater, *k*, made from insulated manganine wire is wound on the gold tube between the T-junction and the channel. The heater was insulated by an air gap from the body of the flow cell and most of the heat evolved is therefore taken up by the liquid flow close to a position where heat is evolved in a reaction experiment.

For comparison purposes a second heater is positioned at *l*. This heater was in good thermal contact with the body of the flow cell and its lid.

With this cell design and the combined heat capacity of the thermopile, 15 min was required to reach a steady state and at normal flow rates this represented 1–1.5 ml of each reactant.

Pumps. The liquids were pumped through the flow cells by means of peristaltic pumps (Perpex). These pumps were found to give a very stable long time flow rate which was not affected by a moderate change in back pressure. During a 48 h pumping period the flow rate change was usually $\leq 0.1\%$.

Connecting tubes. In the present work where only aqueous solutions are investigated soft PVC tubings (1 mm i.d.) were used as connections between pumps, heat exchange unit, flow cell and collection vessel.

Detection and recording of thermopile signal. The differential voltage from the thermopile in the two calorimetric units was amplified by a Keithley 150 B Microvolt Ammeter. The amplified signal was recorded with a Sargent SR recorder. At a low signal level the chart readability was increased by use of a ball and disc integrator.

Electrical calibration circuit. A conventional electrical calibration circuit similar to that described in Ref. 1 was used.

ELECTRICAL CALIBRATION EXPERIMENTS

Position of the calibration heater. Heat evolution at a calibration procedure and at a process under investigation must be closely comparable. It is therefore essential that the position of the calibration heater is such that a certain electrical heat effect will give rise to the same thermocouple signal as an identical heat effect from a process in the flow cell. The following series of experiments were undertaken to demonstrate the properties of the present calorimeter in this respect. Electrical calibrations were performed using the regular heater (*k* in Fig. 2) and the other heater position at *l*. The position of the regular heater is such that essentially all the heat evolved will be taken up by the liquid at a position where a substantial part of the heat will be evolved in a reaction experiment. Heat evolved in the heater position at *l*, however, will be directly transferred to the silver body of the flow cell and from there conducted out to various parts of the cell. Electrical heat effects were similar and flow rates were the same for the two series of experiments. The calibration constant, ϵ , is given in arbitrary units ($\mu\text{cal/sec-recorder response}$).

Results of these experiments are summarized in Table 1. It is seen that there is only a small difference (0.4 %) in the calibration constants obtained with the two heaters. It may thus be concluded that for the present calorimeter it is not very critical at what part of the mixing zone the heat is evolved and, therefore, the heater position should be adequate. Further, it may be assumed that the ϵ -value should not be sensitive to variations in rate of reaction or rate of mixing as long as the process takes place reasonably close to the calibration heater *i.e.* in the mixing zone of the flow cell.

Precision and sensitivity of the calorimeter. From results of repeated calibration experiments it can be concluded that a heat effect measurement under

Table 1. Calibration constants, ϵ , determined with the regular heater and the heater at *l*. Total flow rate was 0.182 ml/min.

Heater	Heat effect, $\mu\text{cal/sec}$	ϵ
Regular heater	331.0	1.874
	331.2	1.875
	330.8	1.878
	330.9	1.877
	331.0	1.872
	330.6	1.878
	Mean	1.876 ± 0.002
Bedded heater	259.6	1.884
	259.6	1.885
	259.4	1.885
	259.5	1.883
	259.6	1.882
	259.3	1.881
	Mean	1.883 ± 0.002

Table 2. Calibration constant, ϵ , at different electrical heat effects. Total flow rate was 0.163 ml/min.

Heat effect, $\mu\text{cal/sec}$	ϵ
88.5	1.879
136.6	1.856
157.4	1.863
241.3	1.870
307.2	1.862
372.9	1.866
Mean = 1.866 ± 0.003	

suitable condition can be made with a precision of 0.1 % at a heat effect of 100 $\mu\text{cal/sec}$ (cf. Table 1) and about 1 % if the heat effect is in the order of 10 $\mu\text{cal/sec}$. A heat effect sensitivity value of 0.1 $\mu\text{cal/sec}$ was evaluated from the stability of the voltage-time curve.

The stability of a steady state value during a 12 h period was usually better than 1 $\mu\text{cal/sec}$.

Calibration constant at different electrical heat effects. Eqn. 4 requires that the calibration constant ϵ is a constant which is not affected by a variation in W_p . In a series of calibration experiments the electrical heat effect was varied whereas other experimental parameters were kept constant. Results are summarized in Table 2 from where it is seen that there is no systematic variation in the ϵ -value with the heat effect. The standard deviation of the mean (± 0.2 %) is well within the expected amplification and recording errors.

Variation in calibration constant with flow rate. In Fig. 3 results are summarized from electrical calibration experiments performed with a constant electrical heat effect but with different flow rates. It is seen that the difference in calibration constant from zero flow rate to a normal total flow rate (about 0.17 ml/min) is less than 3 %. At zero flow rate no heat is transported out

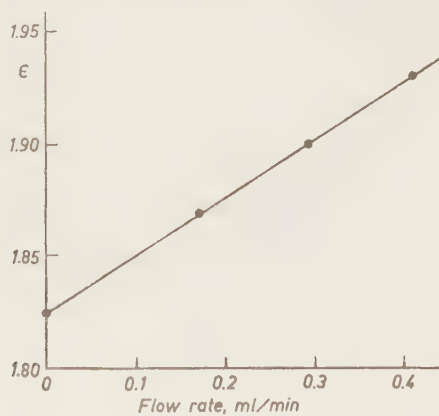


Fig. 3. Variation in calibration constant (ϵ) with flow rate.

from the cell by the liquid flow and $W=W_t$. From the present results it may be concluded that under normal operational conditions 97 % of heat evolved in the flow cell will pass through the thermopile.

ZERO EFFECTS

Heat effect generated by a process under steady state conditions is calculated through eqn. (4), $W_p = \varepsilon \cdot V_p$. Here V_p is the displacement of the thermopile signal from a certain voltage base line to the recorded steady state value. The base line value will vary slightly with the experimental conditions and the following experiments were made to get these variations somewhat explored. The zero effect may be expressed in terms of a heat effect, W'

$$W' = \varepsilon(V' - V_0) \quad (5)$$

where V_0 is the thermopile voltage under apparent equilibrium conditions for the calorimeter and when the flow rate was zero. V' is the thermopile voltage (the "base line value") at the specified liquid flow. V_0 is usually not zero even after a very long equilibration time for the calorimeter. In the present case V_0 was about 1 μ volt.

In eqn. (5) ε is assumed to be identical with that determined at electrical calibration experiments although the correct ε -value for this case might be slightly different.

Zero effect at different flow rates. In Fig. 4 W' is given as a function of total flow rate. The flow was equal in both arms and the liquid was pure water. It is notable that the zero effect is increasingly negative at flow rates up to 0.15 ml/min after which the curve will turn in a positive direction and the W' value will be quite sensitive to variations in the flow rate.

Variation in zero effect with the viscosity of the calorimetric liquid. Fig. 5 shows the variation in the zero effect when at a constant flow rate the calorimet-

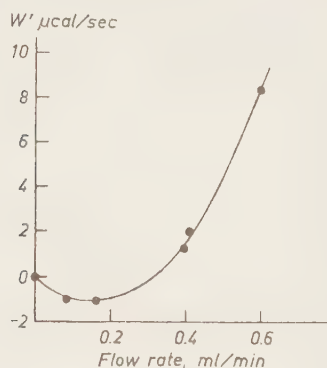


Fig. 4. Zero effect (W') as a function of flow rate.

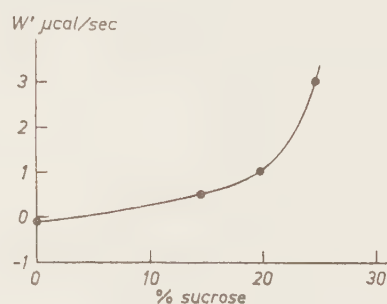


Fig. 5. Zero effect (W') as a function of increasing viscosity of the calorimetric liquid. Solutions of sucrose were pumped through both arms with a total flow rate of 0.165 ml/min.

ric liquid changes viscosity.* Pure water and solutions containing different amounts of sucrose, respectively, were pumped through both of the arms with a total flow rate of 0.165 ml/min. The figure shows that for this particular flow rate the base line value becomes less negative and then positive as the viscosity increases but that the change is small.

From Figs. 4 and 5 it is evident that variation in zero effect with liquid flow rate and liquid viscosity should be considered when small heat effects are measured. The variation expressed as a heat effect, however, is very small and no significant systematic error will arise if the base line value is determined under conditions (flow rate, viscosity *etc.*) reasonably similar to those in the main experiment.

MIXING EFFICIENCY IN THE FLOW CELL

The mixing efficiency is of critical importance at a reaction or dilution experiment in flow calorimetry. During the course of this work several designs of mixing cells were tested and found to be inadequate at experiments where a complete mixing was required.

As a sensitive test for the mixing efficiency "titration curves" for HCl—NaOH solutions were determined. Through one of the arms in the flow cell NaOH solutions of different concentrations were pumped whereas the concentration of the HCl solution through the other arm was kept constant (0.005 or 0.01 M). Changes in heat effect from dilution and mixing of ionic species can be neglected in the investigated concentration ranges. Idellay one would expect a constant heat effect value as long as NaOH is in excess and from the equivalence point the heat effect should decrease linearly to zero as the NaOH concentration approaches zero. Within limits of error the ideal "titration curve" was obtained with the final cell construction.

The first cell investigated had a gold T-piece and a heater applied as in the final design, Fig. 2. The combined flow was taken through a straight gold tube (0.6 mm i.d.) bedded by Woods metal in a copper plate which formed the body of the cell. Mixing in this cell was very poor. In a titration experiment the heat effect did not approach a constant value until the calculated equivalence point had been exceeded by more than a ten fold excess of NaOH.

In another construction a 0.08 ml mixing reservoir was inserted between the T-junction and the gold tube bedded in the copper plate. The mixing efficiency was increased but not sufficiently as shown by the fact that there was required a two fold excess of NaOH to reach the horizontal part of the titration curve. The design of this cell was modified in different ways, *e.g.* inserting constrictions in the mixing reservoir (cell C). These changes resulted in an improved performance which, however, was not very dependable. It was shown that air bubbles entering the flow system were easily trapped in the reservoir and caused a large decrease in the mixing efficiency.

* Constrictions in the flow cell were not identical with those described in the preceding paragraphs.

Model studies which led to the final design of the flow cell showed that for water at room temperature cross section of tubes or channels used in the present type of cell construction should be ≤ 1.5 mm if air bubbles should not be trapped.

HEATS OF DILUTION OF UREA AND SUCROSE

The neutralization experiments referred to above are not suitable as accurate tests for the absolute value of the heat effect measured with the calorimeter. For that purpose it would be necessary to take rigorous and experimentally difficult precautions to avoid CO_2 interference.

More convenient test reactions for a check of the absolute value are provided by dilution experiments involving, *e.g.*, urea and sucrose. One may note, however, that a successive heat of dilution of a compound usually will result in smaller and smaller heat effects. One can therefore expect to obtain an essentially correct heat of dilution value even if the mixing has not been quite perfect. For one of the earlier flow cells tested during this work (cell C) good heat of dilution values were obtained for urea although it was shown by acid base titration experiments that mixing was not adequate.

Urea solutions suitable for dilution experiments with the present calorimeter can be rather dilute and have a low viscosity. They are therefore comparatively easily mixed with water in a flow cell. Sucrose forms a nearly perfect aqueous solution and heats of dilution are therefore very small. In order to get a reasonable heat effect in a dilution experiment it is necessary to use rather concentrated solutions. Such solutions are quite viscous and are not very easily mixed with water in a flow cell without the use of a mechanical stirrer or a similar device. Experiments with flow cell C resulted in very low values for dilution of a 1 molal sugar solution with an equal volume of water (55 % of the calculated value).

In Tables 3 and 4 results are summarized from dilution experiments with urea and sucrose solutions using the final design of flow cell.

In the experiments Mallinckrodt A.R. urea was used without further purification. Sucrose used was a standard sample (≥ 99.95 % purity) kindly supplied by Dr. Tjebbes at Swedish Sugar Corporation, Arlöv. Solutions were freshly prepared before the experiments. Changes in model concentration

Table 3. Heat of dilution of aqueous urea solutions at 25.00°C.

Initial	Molality		Heat effect $\mu\text{cal/sec}$	ΔH cal/mole		$\frac{\Delta H_{\text{exp}}}{\Delta H_{\text{calc}}} \times 100$
	Initial	Final		Experimental	Calculated	
	0.916	0.447	40.38	36.54	36.34	100.6
	1.053	0.512	52.16	41.31	41.28	100.1
	1.279	0.619	74.68	49.16	49.10	100.1
	1.456	0.700	94.29	55.28	55.15	100.2
	1.659	0.806	119.49	61.60	60.78	101.3
	1.957	0.933	158.70	70.25	70.64	99.4

Table 4. Heat of dilution of aqueous sucrose solutions at 25.00°C.

Initial	Molality		Heat effect $\mu\text{cal/sec}$	$-\Delta H$ cal/mole		$\frac{\Delta H_{\text{exp}}}{\Delta H_{\text{calc}}} \times 100$
	Initial	Final		Experimental	Calculated	
	0.516	0.250	22.45	34.49	34.37	100.3
	0.731	0.347	43.22	48.81	48.77	100.1
	0.752	0.375	41.45	47.40	47.75	99.3
	1.252	0.598	106.42	79.70	79.50	100.3

were calculated from measured volume/time flow rates and known densities for solutions of urea,⁷ and sucrose,⁸ respectively.

The obtained heat of dilution values are compared with data calculated from the expressions given by Gucker *et al.* for the apparent heat content of urea⁹ and sucrose¹⁰ in aqueous solution. All experiments were made at 25.00°C.

From the tables it is seen that in all cases there is a good agreement between determined and calculated values. The differences, usually less than 1 %, are judged to be well within the combined limits of uncertainty. Recently¹ the heat of dilution of a 0.2 molal sucrose solution with twice the amount of water was determined with the aforementioned batch micro calorimeter and found to be 1.4 % higher than the calculated values. With the same batch calorimeter the heat of dilution of a 1.05 molal urea solution with twice the amount of water has been determined and was found to agree within 0.2 % with the calculated value. Stoesser and Gill recently reported⁶ a value 3 % lower than the calculated value * for the dilution of a 1 molal urea solution with an equal volume of water.

COMPARISON BETWEEN THE BATCH AND THE FLOW MICRO CALORIMETER

The present flow calorimeter is based upon the design of the earlier reported batch micro calorimeter. Some concluding remarks where the two calorimeters are compared will be given here.

Operational procedure. The experimental procedure with the batch calorimeter is very simple. The present flow method, however, is by principle completely automatic and does not require any equilibration time. The flow method will therefore offer distinct advantages in particular if the calorimeter is used for extensive series of measurements or if it is used as an analytical tool.¹¹

Sensitivity and accuracy. The sensitivity in terms of thermopile voltage at a given heat effect is essentially the same for the two constructions. The batch calorimeter will in practice be a more sensitive instrument as heat from the combination of about 5 ml of calorimetric liquids is evolved as a

* A calculation error appears in Ref. 6. The value calculated from the data by Gucker *et al.*⁹ for the process referred to in Ref. 6 should be 3.74 mcal.

pulse (for a fast process). In the flow version corresponding heat is continuously evolved during about half an hour.

It is judged that the present flow method can be developed to give a considerably higher sensitivity whereas for the batch version possible systematic errors¹ might make an increased sensitivity of little practical value.

Reaction time. The batch calorimeter is suitable for reactions ranging from instantaneous processes to reactions with a duration of several hours. For the flow calorimeter it is for a normal operational procedure required that heat should be evolved in the mixing zone, *i.e.* the reaction time should be short compared to the retention time for the liquid in the flow cell. For the present flow cell and with a normal flow rate the retention time is about 5 min. It is considered that any reaction shorter than 1 min is suitable for precise measurements. For slow reactions it is possible to perform "stopped flow" experiments where a pulse of the calorimetric liquids is pumped into the flow cell in which the reaction mixture is kept until all the heat is given off. However, the batch calorimeter will give considerably more precise results than a stopped flow experiment.

The flow method will offer great advantages compared to the batch procedure when steady state reactions, like enzymatic processes with substrate saturated enzyme, are studied. This is in particular apparent when the calorimeter is used as an analytical instrument.¹¹

Acknowledgement. This project has been financially supported by the Swedish Council for Applied Research.

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FLOW MICRO-CALORIMETRY AS AN ANALYTICAL TOOL IN MICROBIOLOGY

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(Received July 3, 1969)

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INTRODUCTION

Practically all processes—they may be of physical, chemical or biological nature—are accompanied by heat effects. It is possible to determine accurately very small heat quantities, and one can follow heat evolution during long periods of time. Heat measurement methods—calorimetric methods—are therefore of a potential interest as an analytical principle for all kinds of chemical and biological analysis.

With the exception of various DTA methods, one may conclude, however, that calorimetric methods have not found a wide use as analytical tools. The main arguments against calorimetric analytical methods have been their low specificity, a usually rather complicated technique, and, for systems in solution, a moderate sensitivity compared to, e.g., spectrophotometric methods.

As a result of recent developments in microcalorimetry the situation has become more promising. Recently (Monk & Wadsö, 1968) a flow microcalorimeter was developed from an earlier batch version (Wadsö, 1968). Besides being a thermodynamic instrument this latter calorimeter has proved useful as an analytical instrument. It is characterized by a high sensitivity and a very simple experimental procedure as demonstrated in determinations of enzyme activities and of substrate concentrations by enzymatic methods (Monk & Wadsö, 1969).

As is the case for any calorimetric method, the specificity is low; for complex processes it is, however, sometimes advantageous to use a nonspecific analytical method which gives a gross value for the process.

Calorimetric methods do not require transparent solutions, as do many analytical procedures, and this is of particular importance in such fields as microbiology.

Calorimetric methods have been utilized for a long time in microbiological research (see, e.g., Calvet & Prat, 1956; Prat, 1962; Belaich *et al.*, 1968; Kalakoutsii & Pozharitzkaja, 1968; Forrest, 1969), but the main emphasis has usually been to correlate observed heat effects with thermodynamic data. In such work both macro- and micro-

systems have proved to be useful, but no flow technique seems to have been used hitherto. The purpose of this report is to discuss the use of flow micro-calorimetry as an analytical tool in microbiology.

EXPERIMENTAL

Calorimeter

The flow calorimeter used has been described in detail elsewhere (Monk & Wadsö, 1968). It is a twin calorimeter using the heat leakage principle. Heat evolved in the reaction cell is conducted out to a surrounding heat sink. The arrangement is such that the heat exchange takes place through a thermopile made from semiconducting material, and the heat flux will be observed as an electrical potential which is amplified and recorded. In the bacterial experiments described a flow-through cell (Monk & Wadsö, 1969) with an internal volume of 1.5 ml is used.

The analytical technique is illustrated by growth experiments with *E. coli* (Astra strain 1339).

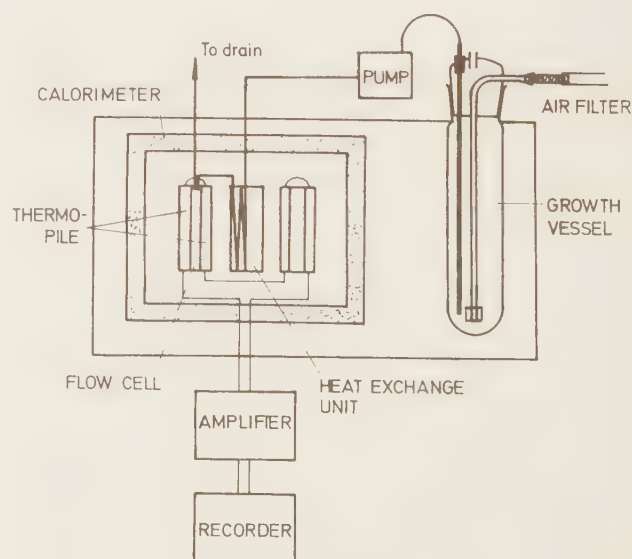


Fig. 1. Principal arrangement of the apparatus for flow micro calorimetric studies of bacterial growth.

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Fig. 2. Thermograms from the growth of *E. coli* on 'Antibiotic Medium 3'. The curves show the calorimetrically measured heat effect versus time. Fig. 2b also shows the corresponding turbidimetric curve

Growth Vessel

The growth vessel was a 1-l glass flask with a ground joint stopper fitted with a glass-filter aerator and a steel tube connected to the pump. The flask was submerged in the thermostatically controlled water bath containing the calorimeter.

Procedure

The bacterial suspension to be investigated was pumped from the growth vessel by a peristaltic pump (Perpex) to the heat-exchange unit in the calorimetric block and finally through the calorimetric cell, Fig. 1. After passing through the calorimeter the suspension was in these experiments taken to drain, but could be returned to the growth vessel.

The liquid flow rate was 40 ml/h, and the thermostat temperature was 28.0 °C. Before an experiment the pump and the calorimetric tube system were cleaned and sterilized by pumping through them 0.1-M NaOH followed by sterile water.

The experiments were started by pumping sterile growth medium through the calorimeter until a stable baseline was obtained (ca. 15 min), after which 20 ml of inoculum was added to the medium in the growth vessel. The inoculum was prepared by incubating 20 ml of the same growth medium for 20 h before the calorimetric experiment.

For the turbidimetric measurements (Fig. 2b) a Klett-Summerson colorimeter was used. Since turbidity was low, corrections for deviations from Beer's law were not made.

Results

Results from measurements on a complex growth medium are shown in Fig. 2. 'Antibiotic Medium 3' (Difco Laboratories) was used: 0.15% beef extract, 0.15% yeast extract, 0.5% peptone, 0.1% dextrose, 0.35% NaCl, 0.361% K_2HPO_4 and 0.132% KH_2PO_4 .

The two thermograms started with a very profiled heat effect curve, and after about 6 h there was a thermal lag phase with nearly zero heat effect. An isolated smooth peak followed, after which there was an extended lag phase, followed by a comparatively large heat effect.

The two experiments were made under similar conditions, and most of the characteristics of the two complex thermograms are obtained in both experiments. It may be noted, however, that the time interval between characteristic points varies slightly, e.g. the shoulder, A, and the minimum, B, following it (Fig. 2a) correspond to the two minima in Fig. 2b.

Fig. 3. Thermograms from the growth of *E. coli* on 0.25% glucose solution, salt medium and different concentrations of yeast extract.

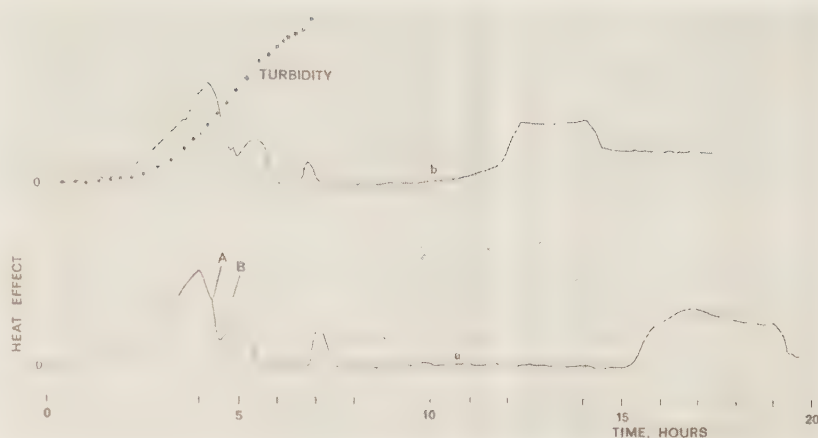


Fig. 2b also shows the corresponding turbidimetric curve (log absorbance versus time). A two-stage process is observed, but the calorimetric curve is considerably more characteristic.

The different steps of the thermograms in Fig. 2 are believed to reflect induction periods necessary for the organisms to adapt themselves to the available components of the growth medium.

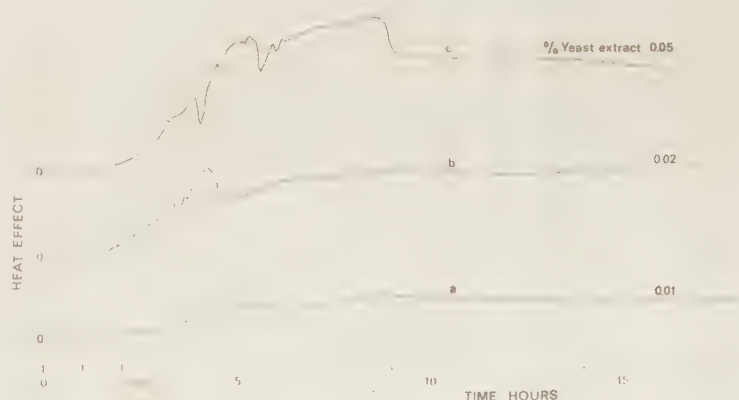
The *E. coli* strain used has rather extensive requirements for growth factors, as shown by the results illustrated in Fig. 3. The bacteria were grown on an 0.25% glucose solution to which was added salts (0.135% KH_2PO_4 , 0.1% NH_4PO_4 , trace elements) and different amounts of yeast extract (Bacto Yeast Extract, Difco Laboratories); the initial pH was 7.0.

The heat effect increased with the concentration of yeast extract, and as for the experiments recorded in Fig. 2 one can note a highly profiled initial part of the thermograms; in particular for those with a high concentration of yeast extract (Fig. 3). In Figs. 3b and 3c the similarity in shape of the initial parts of the thermograms can be recognized, although the resolution varied with the concentration of yeast extract.

The initial part of the thermograms in Fig. 3 is tentatively believed to be related to the catabolism of the yeast extract. Fig. 4 shows a thermogram from an experiment where the bacteria were grown on 0.05% yeast extract and salt medium but no glucose. A highly profiled curve was obtained, and after about 6 h abruptly decreased and then slowly descended to zero heat effect.

DISCUSSION

Flow microcalorimetry offers several advantages over batch techniques, in particular when applied to analytical problems. The experimental procedure is very simple, and essentially no equilibration time is required.



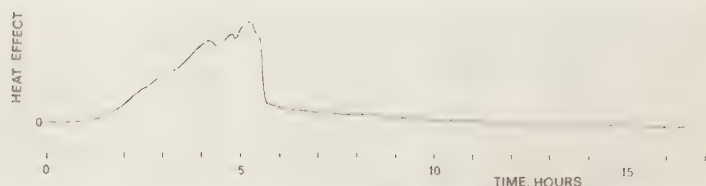


Fig. 4. Thermogram from the growth of *E. coli* on 0.05% yeast extract and salt medium

Repeated treatment of the batch culture, e.g. addition of growth factors or antibiotics, can be made without disturbing the calorimetric measurement.

It is further possible to examine continuous processes, and the method will lend itself to automatic analytical procedures. It also seems possible to use flow calorimetry for control and regulation of biotechnical processes.

Every characteristic of complex thermograms, like those in Figs. 2 and 3, has biochemical or biological significance, and therefore the method should be a useful analytical tool for discovering unknown effects and for proper timing of more specific analytical techniques to be applied on the batch culture.

One important problem at any calorimetric procedure for investigating aerobic bacterial growth is to provide a suitable oxygen concentration at the site of the calorimetric measurement, i.e., in a batch calorimetric vessel or, as here, in the flow cell. If the thermal signal is low, artefact signals from vaporization are easily introduced if a two phase system is used. In systems of the present type there will be additional disturbances from gas bubbles transported with the liquid flow which will displace part of the active volume of the flow cell, usually in a random way.

In the experiments reported here no gas bubbles were introduced into the calorimeter. The time required for the liquid to reach the calorimetric cell from the growth vessel was about 3 minutes, and one may assume that the oxygen pressure was low in the calorimetric cell.

It is, however, believed that the process recorded was not of anaerobic growth, because the signal was reduced to about half if the flow was stopped.

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The Use of Microcalorimetry for Bacterial Classification

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BACTERIAL GROWTH processes studied under anaerobic conditions in various batch calorimeters have invariably shown smooth simple thermograms whether complex or defined media have been used (Forrest, 1972).

Using a flow calorimetric method (Monk & Wadsö, 1968) it was shown by Delin, Monk & Wadsö (1969) that highly characteristic growth profiles were obtained for *Escherichia coli* grown aerobically on complex media and it was concluded that such thermograms are potentially useful for detecting the growth of, and characterizing, microbes. Recently, Boling, Blanchard & Russel (1973) have shown the possibility of characterizing different bacteria from their thermograms. This is possible because the observed thermograms are complex and differ considerably in detail from one organism to another under nominally identical conditions. No explanation has been proposed either for the complexity or the observed differences, while the variation with different experimental conditions such as the availability of oxygen have not been investigated. However, Binford, Binford & Adler (1973) using a flow microcalorimetric technique for antibiotic sensitivity tests on bacteria noted different heat effect patterns for cultures grown at different oxygen concentrations.

Methods

In this work the growth patterns of *Streptococcus faecium*, *Strep. faecalis* var. *liquefaciens*, a β -haemolytic *Streptococcus* belonging to Lancefield group B (*Strep. agalactiae*) and of *E. coli* 281 have been determined at different oxygen concentrations. The 3 *Streptococcus* spp. were identified at the Dept. of Medical Microbiology, University of Lund. The *E. coli* strain was obtained from the Dept. of Microbial Ecology, University of Lund, and the identification number refers to the Dept. of Bacteriology, University of Wisconsin, Madison. The organisms were grown aerobically and anaerobically on a complex medium containing 1% each of glucose, yeast extract, peptone and sodium citrate, sterilized at 121° for 15 min. Inocula were prepared by growing the organisms on the same medium overnight and then using this suspension at the 1% level.

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Fig. 1. Thermograms obtained under different oxygen concentrations. In flow experiments growth was aerobic; in stopped flow and in ampoule experiments growth was anaerobic. (a) *Strep. faecium*, (b) *Strep. faecalis* var. *liquefaciens*, (c) β -haemolytic *Streptococcus* sp. belonging to Lancefield group B (*Strep. agalactiae*), (d) *E. coli* 281.

The thermograms of growth were recorded by a flow calorimeter (Monk & Wadsö, 1968) having a cell volume of 0.7 ml and maintained at 37°. The flow cell used was identical to the flow-through cells used with the LKB flow microcalorimeter 10700-1. Aerobic conditions were maintained by flow re-circulation to an air gassed reservoir and anaerobiosis was obtained in a comparable sample of growing organisms by stopping the flow when the measuring cell had filled with inoculated medium. The gold tubing of the cell is impermeable and the organisms rapidly remove the last traces of oxygen.

In one experiment an ampoule loaded microcalorimeter (Wadsö, 1973) was used to record the anaerobic thermogram, 0.5 ml of inoculated medium being enclosed in a stainless steel ampoule.

Results and Discussion

The thermograms (Fig. 1) show that the characteristic profiles of bacterial growth are strongly related to the oxygen status of the growth medium. *Strep. faecium* and the β -hemolytic *Streptococcus* sp. have similar properties in this respect. The anaerobic pattern for *Strep. faecalis* shows the same simple bell shaped curve as obtained for the *Strep. faecium* strain and is similar to that for the β -haemolytic *Streptococcus* sp. However, an entirely different thermogram is found for *Strep. faecalis* under aerobic conditions. In some flow experiments with this organism the growth reservoir was gassed with N₂. The thermograms then had 3 phases which, however, were much less marked than those shown in Fig. 1(c). In these cases it is likely that a limited amount of oxygen had penetrated the Teflon and silicone tubings of the calorimeter flow line but not enough to make the growth conditions fully aerobic in the calorimetric vessel. The 2 thermograms shown for *E. coli* [Fig. 1(d)] are very different from each other and the anaerobic profile is markedly different from corresponding curves for the *Streptococcus* spp.

Every process produces heat; it is therefore essential to define the system so that the reactions which take place can be characterized accurately. In particular, oxidation effects are strongly exothermic so that quantities of oxygen undetectable by chemical analysis can produce significant amounts of heat. Hence the use of thermogram 'fingerprints' for identification of different organisms will depend on exact definition of experimental conditions. As seen from this work there is a marked difference between flow and stopped flow experiments using the same instrument. One may expect that if the calorimetric vessel in a batch instrument is made from plastic material the oxygen permeability through the vessel may affect the shape of the thermogram.

This work has been supported by the Swedish Board for Technical Developments. The assistance of Mrs K. Ljungholm is gratefully acknowledged. We also thank Dr F. Christensen for identifying the *Streptococcus* spp.

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IV

3.5 Calorimetric Studies of Lactic Acid Bacteria and the Effect of 2,4-Dinitrophenol on their Catabolic Regulation

P. Monk, W. Forrest, I. Wadsö

INTRODUCTION

It has been amply demonstrated that the ability of non-proliferating suspensions of many lactic acid bacteria to degrade glucose is a well defined property (1,2,3). Thus suspensions prepared in the same way may reliably be expected to have kinetic properties reproducible to better than 2 % (2), more stable than most enzyme preparations.

Oxygen may modify the fermentation of lactic acid bacteria and large heat effects have been observed when Streptococci grow in the presence of oxygen causing their thermograms to become complex (4).

Metabolic inhibitors and uncouplers have extensive influence on glycolysis in organisms carrying out oxidative phosphorylation. DNP is an uncoupler that is widely employed and its mode of action in this area is that of uncoupling catabolism from oxidative phosphorylation. It is also generally considered to affect only oxidative phosphorylation and not anaerobic glycolysis (5).

The relationship between four Streptococci and oxygen has been examined calorimetrically and in one case correlated with product formation. The effect of oxygen and DNP on catabolic regulation in nongrowing and growing cells has been investigated.

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EXPERIMENTAL

Calorimetric experiments were carried out with a heat conduction flow microcalorimeter fitted with a flow through cell (6). Experimental details were the same as reported elsewhere (4).

RESULTS

Experiments with Washed Suspensions without DNP

When a washed suspension of Streptococci in phosphate buffer is pumped through a flow calorimeter a steady state baseline is established slightly above that for buffer alone. This heat effect is a measure of the small endogenous metabolism of the cells (7). On addition of glucose to the bacterial suspension being pumped to the calorimeter, the heat effect increases to a new steady state which includes the liberation of heat from catabolism of the glucose and an oxidative heat effect. On stopping the flow the established steady state is maintained for a short time, the heat effect then slowly decreases linearly and then exponentially to establish a new steady state of anaerobic glycolysis. Re-starting the flow causes the heat effect to increase to the initial steady state.

The bacterial suspension brings about the anaerobic state by utilization of the dissolved oxygen which was previously supplied by the flowing suspension. The time taken to establish the new steady state is dependent upon the bacterial cell density of the suspension located in the calorimeter cell and the oxygen status of the suspension at the time the flow was stopped.

A relationship can be established between the anaerobic (stopped flow) and aerobic (flow) heat effects for Streptococci, catabolizing glucose. By plotting the aerobic heat effect against the anaerobic heat effect for each organism a linear relationship is obtained. This relationship is reproducible for each strain and found to be independent of decreased ability to catabolize glucose when examined up to thirty hours after harvesting the cells. Different activities are obtained by varying the cell density of the suspensions.

The anaerobic heat effect is a quantitative measure of the rate of glucose degradation to lactic acid in a homolactic fermentation (2). The aerobic heat effect is the sum of the oxidative component of the suspension and the decreased anaerobic heat effect due to the oxidation of a fraction of a metabolic intermediate.

The reproducibility of the measurements demonstrates the ability of the technique to measure the oxidative ability of Streptococci and to differentiate between them on this basis.

The intermediate undergoing oxidation can be determined as pyruvate by catabolism in the presence of semicarbazide and degradation of added pyruvate.

Experiments with Washed Suspensions with 2,4-Dinitrophenol

When the molar turnover of glucose is measured anaerobically and aerobically no difference is observed in the rate of degradation, but the amount of lactate produced aerobically differs from a homolactic fermentation. This is due to the aerobic oxidation of pyruvate. The deviation from a homolactic fermentation is variable depending upon the organism.

If DNP is added to a suspension catabolizing glucose the rate of glycolysis increases and if the system is anaerobic the increased turnover of glucose appears as lactate and the fermentation is homolactic. The increased heat effect during stimulation can be correlated with increased lactate production.

When DNP is added over a range of 0-400 $\mu\text{mole/l}$ to a suspension catabolizing glucose, the heat production both anaerobically and aerobically increases to a maximum rate at about 200 $\mu\text{mole/l}$. The increased heat production can be as high as 70 % of the basal rate.

Examination of the rate of glucose degradation immediately after the addition of glucose to a suspension with and without DNP, shows cells without DNP to regulate their catabolic activity to a lower rate within a

few minutes after glucose addition. Cells with DNP however continue at the initial elevated rate.

GROWTH IN THE PRESENCE OF 2,4-DINITROPHENOL

Streptococci grown in a complex medium containing limiting glucose, with and without DNP, exhibit the same specific growth rate, but the cell yield is lower in the presence of DNP, possibly due to catabolic regulation being affected and glucose being degraded faster than the energy generated can be coupled to anabolic processes.

DISCUSSION

It is well established that different strains of Streptococci are micro-aerophilic and the strains exhibit differences in their ratio of oxidative to anaerobic heat effect. The precision with which this ratio can be measured suggests that this could be a useful taxonomic feature, being an extension of the suggestion already made that glycolytic activity should be used in chemical taxonomy of Streptococci (3).

Oxygen does not effect the rate of glycolysis, but does change the balance of products formed. In one experiment glucose gave 1.67 moles of lactic acid instead of two moles expected from a homolactic fermentation.

It is likely that the oxidation of pyruvate would give rise to acetate (8,9) which is supported by results of the following thermochemical calculations.

It is assumed that the steady state process under aerobic conditions can be described by



The anaerobic state exists when $x = 0$.

Taking into consideration pKa values and enthalpies of ionization (10), the enthalpy change for reaction (1) was calculated from literature data (11) to be $\Delta H(\text{phosphate buffer, pH} = 6.2) = -(117 + 509x)$ kJ/mole glucose. For the anaerobic condition ΔH will be -117 kJ/mole glucose. As the rate of glucose degradation is not affected by the presence of oxygen and the ratio between the heat effect for aerobic and anaerobic conditions, R , is thus given by

$$R = \frac{117 + 509x}{1}$$

For the culture studied we observed this value to be $R = 2.35$ leading to a value for $x = 0.31$ mole. Experimentally we found that $2 - x = 1.67$ or $x = 0.33$ mole.

Cells catabolizing glucose in the absence of DNP are observed to effect a transition from a rapid initial rate to a lower rate. The transition is rapid, and has the characteristics of positive feedback so that regulation of glycolysis in this uncoupled condition appears to consist of the organism glycolyzing at a minimum rate. In the presence of DNP the transition does not occur and the cells retain their initial rapid rate of catabolism; the DNP inactivates the normal feedback regulation. The catabolism of glucose is already completely uncoupled in the washed non-growing suspension and the observed effect of DNP, normally considered as an uncoupler (5), obviously cannot be explained in terms of uncoupling.

In growth the addition of DNP was found to increase the rate of glycolysis similarly to the effect of non-growing suspensions, but the specific growth rate of the organism was not affected. The rate of synthesis of new biomass by each existing unit of biomass is thus unchanged so that the same amount of biologically available energy is coupled to synthesis either in the presence or absence of DNP.

The effect of DNP cannot be localised more precisely from the present experiments than to an interaction occurring before the production of endogenous pyruvate in the Embden-Meyerhof pathway, since its addition increases only the rate of glycolysis and lactate production; aerobic

oxidation of pyruvate is unaffected by DNP. The maximum effect of DNP is a stimulation of anaerobic glycolysis by a factor of about 1.6. This is closely the same as the increased rate of glycolysis found during transient states in physiologically normal cells (12). The great difference in the effect of DNP is its concentration dependence, with a progressive increase to a maximum as the concentration is raised, compared with discontinuous changes observed in physiologically normal cells. It appears then that DNP is progressively removing the feedback control which reduces the rate of glycolysis to a minimum level, while having no other effect on the products of metabolism. Calorimetric observations are consistent with the hypothesis that no metabolic changes occur, except for increased lactate production.

A more detailed discussion will be reported elsewhere.

ACKNOWLEDGEMENTS

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V

Calorimetric identification of several strains of lactic acid bacteria

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SUMMARY. A microcalorimetric method has been developed for the identification of 9 representative strains of lactic acid bacteria used in the dairy industry. A chemically defined medium containing several carbohydrates gave reproducible and characteristic growth patterns. The technique is proposed as a rapid alternative method for the identification of bacteria selected for commercial use.

With the development of modern calorimeters, the application of calorimetry to the study of biological systems and in particular microbial metabolism has been shown to be a useful technique (Spink & Wadsö, 1976).

A calorimetric record of microbial growth, a thermogram, can be characteristic of a culture when grown in a suitable medium under controlled conditions, and may be used to identify a large number of closely related strains (Russell *et al.* 1975). The application of microbial thermograms to applied problems is at present limited by a poor understanding of the cause of heat profiles, while growth measured by other methods may not show the variable events recorded by a thermogram. However, application to a specific area should be feasible if the requirements of reproducibility and simplicity of operation could be satisfied.

The aim of the present study has been to explore the use of a simple microcalorimetric technique for the identification of lactic acid bacteria commonly used in the dairy industry. Different growth conditions have been used and thermograms compared for 9 strains representing the genera *Streptococcus*, *Lactobacillus* and *Leuconostoc*. A synthetic medium has been devised to allow differentiation of the organisms on the basis of their varied fermentative ability.

MATERIALS AND METHODS

Micro-organisms

All the bacteria used in this work were gifts from the Swedish Dairy Association, Malmö. The cultures were *Streptococcus cremoris* 703; *Str. cremoris* 609 RW, *Str. lactis* 38, *Str. diacetylactis* 65, *Leuconostoc cremoris* 80, *Str. thermophilus* CNRZ 302, *Lactobacillus bulgaricus* CNRZ 86, *L. helveticus* CNRZ 303, and *L. acidophilus* 1748.

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Stock cultures

The cultures were maintained by growth in 10 % (w:v) reconstituted low heat skim-milk powder (Scandmilk, Sweden) autoclaved for 10 min at 121 °C. After incubation for 24 h at 30 °C for the mesophilic strains and 37 °C for the thermophilic strains, the milk medium was inoculated with 2 % (v:v) of the grown culture and stored at -22 °C without prior incubation. Yeast extract 0.5 % (w:v) (Difco Laboratories, Detroit, Michigan, USA) was added to the milk medium used for *Leuc. cremoris* and *L. acidophilus*.

Complex medium

The medium contained/l: lactose 2 g, glucose 2 g, tri-sodium citrate 1 g, tryptone, peptone and yeast extract, 5 g each (Difco Laboratories), MgCl₂ 0.1 g, NaH₂PO₄ 8.5 g, and KH₂PO₄ 2 g. The pH of the medium was adjusted to 7.0 with 2 M-KOH solution and autoclaved at 121 °C for 10 min.

Defined medium

The medium contained the individual amino acids as used by Reiter & Oram (1962) and the vitamins and nucleotides used by Ford, Perry & Briggs (1958). To each l of this medium was added β -disodium glycerophosphate 8 g, K₂HPO₄ 3 g, KH₂PO₄ 3 g, Na acetate 0.6 g, Tween 80 1 ml, lactose 0.5 g, glucose 1 g, galactose 2 g, sucrose 2 g, maltose 2 g, raffinose 2 g, trehalose dihydrate 2 g, and ascorbic acid 0.5 g. The medium components except the vitamins were dissolved in distilled water and the pH adjusted to 7.0 with 2 M-KOH. The volume was made up to 900 ml and the medium autoclaved at 121 °C for 10 min. An aqueous solution of the vitamins as a 10-fold concentration was sterilized by filtration through a Millipore filter (pore size 0.22 μ m) and 100 ml was added to the heat sterilized medium.

Preparation of inoculum

The frozen stock cultures were incubated at either 30 or 37 °C and sub-cultured daily into the reconstituted milk medium before being used as an inoculum. The number of sub-cultures required to obtain a reproducible thermogram depended upon the time of storage and the particular strain. With storage times of less than 1 month, 5-6 sub-cultures were required for the strains of *Leuc. cremoris* and *Str. thermophilus* while 2-3 sub-cultures were sufficient for the other cultures. For the calorimetric identification, 0.1 ml inoculum from a 20-h culture grown in the milk medium was added to 5 ml of the test medium. Volumes of 1 ml were generally used for the calorimetric measurements.

Calorimetric measurements

Twin microcalorimeters of the heat conduction type were used. Their design and the experimental procedure has been described in detail elsewhere (Wadsö, 1974). The samples were enclosed in hermetically sealed stainless-steel ampoules previously sterilized at 160 °C for 1 h. The ampoules were allowed to temperature equilibrate before insertion into the ampoule holder in the thermopile zone. The thermograms were recorded under static and anaerobic conditions. Over a 24-h period the baseline

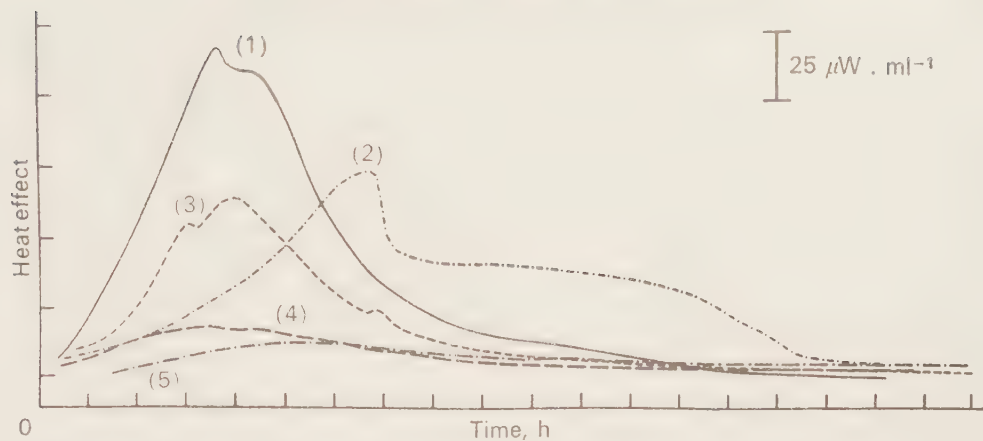


Fig. 1. Growth in complex medium at 37 °C of *Streptococcus diacetylactis* 65 (1), *Lactobacillus bulgaricus* CNRZ 36 (2), *Str. cremoris* 703 (3), *Str. cremoris* 609 RW (4), and *Str. lactis* 38 (5). Time of inoculation is shown as zero hours and the increase in heat effect in units of 25 $\mu\text{W ml}^{-1}$.

stability was $\geq 1 \mu\text{W}$ and the half-decay time of the calorimeter was about 60 s. The thermograms did not require dynamic correction.

Although the optimum growth temperatures for the cultures varied, in order to simplify the experimental procedure an attempt was made to use the same calorimetric temperature for all the strains. It was found that 37 °C was satisfactory in all cases except for *Str. cremoris* 609 RW which required an incubation temperature of 30 °C to obtain rapid growth.

RESULTS AND DISCUSSION

Fig. 1, shows the thermograms obtained with 5 different strains grown in the complex medium at 37 °C. Three of the strains *Str. cremoris* 703, *Str. diacetylactis* 65, and *L. bulgaricus* CNRZ 303 grew well and could be differentiated while *Str. cremoris* 609 W and *Str. lactis* 38 showed poor growth. The complex medium gave reproducible thermograms when the experimental conditions were kept strictly constant. It was observed that the thermograms were significantly affected by changing the type or batch of yeast extract or peptone used in preparing the medium. Thus, commercially available medium does not allow sufficient control of medium composition to obtain reproducible thermograms. To obtain satisfactory reproducibility it was found necessary to use a synthetic medium, and differentiation between bacteria was aided by incorporating a variety of carbohydrate energy sources. The total concentration of carbohydrates in the medium was not sufficient to allow complete growth and the proportions of the individual carbohydrates were adjusted to limit growth on the preferred energy substrates while giving a profiled thermogram. The β -disodium glycerophosphate was incorporated to improve buffer capacity.

Typical results from the experiments using the chemically defined medium are shown in Figs 2–4. For each organism 3 thermograms are shown which were obtained at independent experiments. For *Str. cremoris* 609 RW, Fig. 2, the growth is seen to be poor at 37 °C and for this strain measurements were also made at 30 °C.

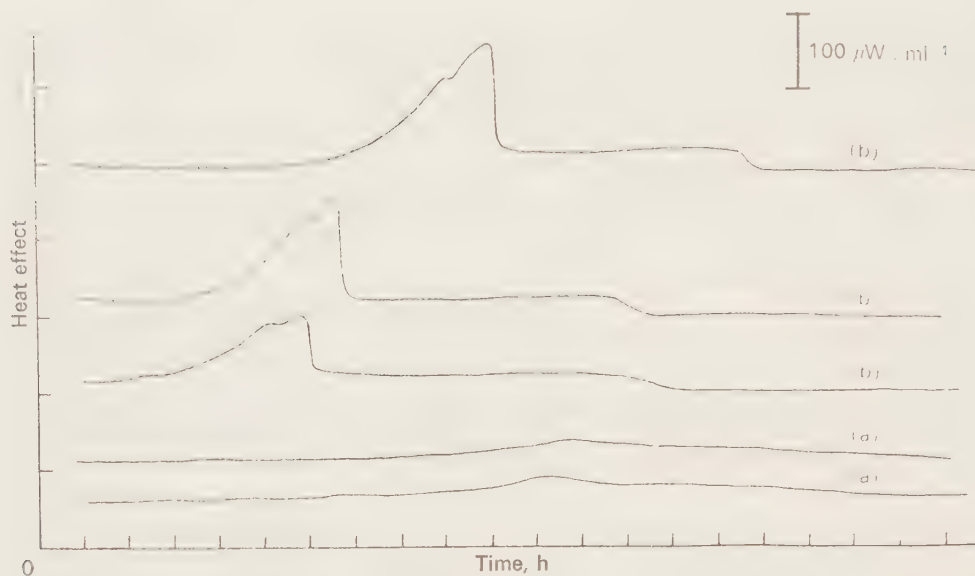


Fig. 2. Growth of *Streptococcus cremoris* 609 RW in defined medium at 30 °C (a) and 37 °C (b). Time of inoculation is shown as zero hours and the replicate thermograms are displaced for clarity. The increase in heat effect is shown in units of $100 \mu\text{W ml}^{-1}$.

It is seen that there is a clear difference in growth pattern between all 9 strains investigated. It is also noted that there is better differentiation than that obtained with the complex medium, Fig. 1.

For each strain the reproducibility is qualitatively good. However, there are significant differences between the time of appearance of the characteristic points (peaks, shoulders and minima), and the corresponding heat effect values. The number of viable cells in the inoculum was not adjusted at each measurement and differences in lag time could result in the displacement of characteristic events. The concentration of the growth limiting substrate was the same at each experiment. In replicate experiments both the total cell growth and heat generated by catabolism of each substrate would be the same. Differences in the maximum heat effect observed would then be related to variable growth rates, a faster growth rate giving a higher heat effect. In a few cases not reported in Figs 2–4, non-characteristic thermograms were obtained.

The temperature of storage of starter cultures is known to affect their performance in milk fermentations (Gilliland, 1977). In this work the requirement to sub-culture some strains several times after freezing to obtain reproducible thermograms emphasizes this observation. It is of interest to note the variability between strains and the ability of thermograms to follow the recovery of the complement of fermentative ability. This technique could be used to investigate the effect of time and conditions of storage on the subsequent metabolic properties of starter cultures.

The use of thermograms for the identification of lactic acid bacteria selected for use in the dairy industry appears to satisfy the requirements of a rapid and reproducible method. Although thermograms of microbial growth are sensitive to many



Fig. 3. Growth of mesophilic strains in defined medium at 37 °C. *Streptococcus cremoris* 703 (a), *Str. lactis* 38 (b), *Str. diacetylactis* 65 (c), and *Leuconostoc cremoris* 80 (d). Three replicate thermograms are shown for each strain and are displaced on the response axis for clarity. Time of inoculation is shown as zero hours and the units of increase in heat effect are shown on each figure.

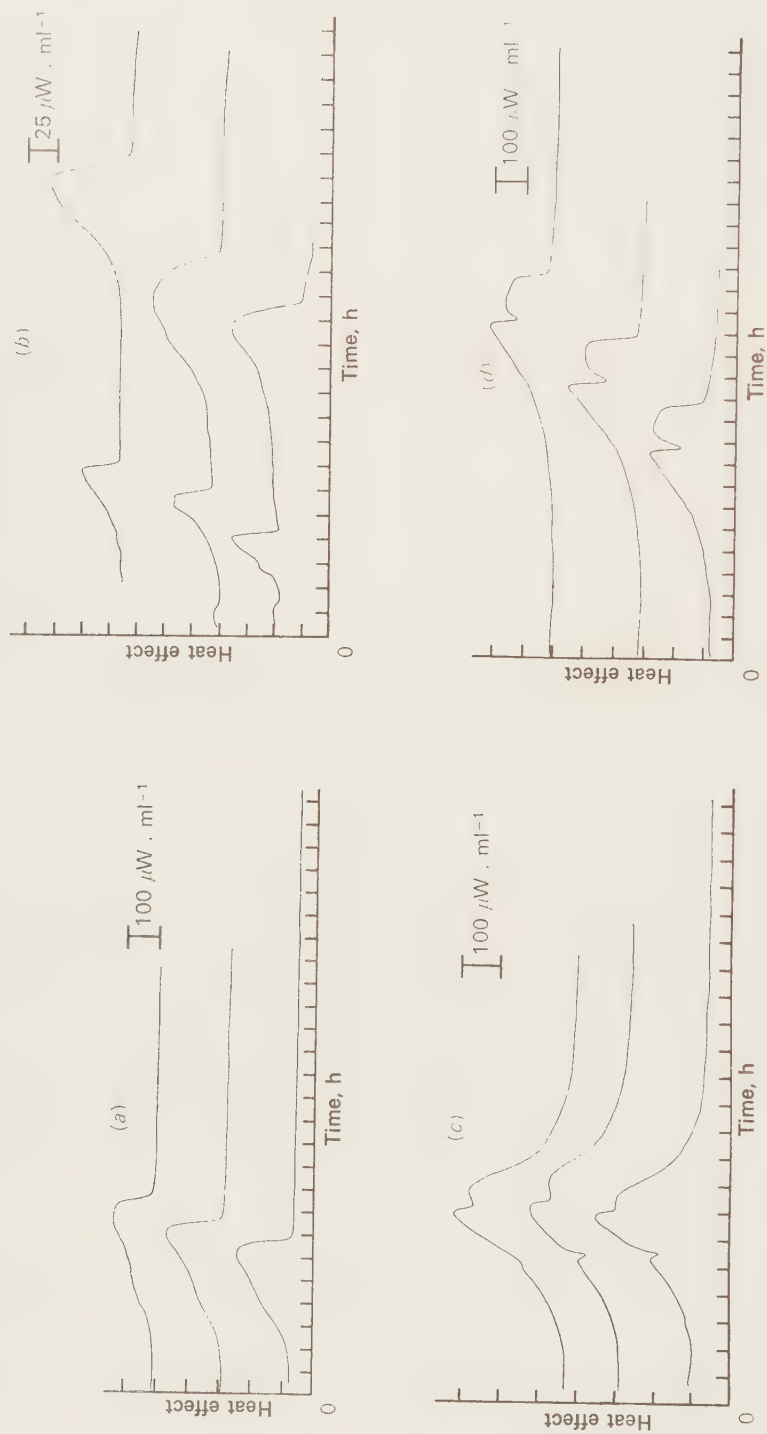


Fig. 4. Growth of thermophilic strains in defined medium at 37 °C. *Streptococcus thermophilus* CNRZ 302 (a), *Lactobacillus bulgaricus* CNRZ 36 (b), *L. helveticus* CNRZ 303 (c), and *L. acidophilus* 1748 (d). Three replicate thermograms are shown for each strain. The notation is the same as for Fig. 3.

experimental parameters including medium composition, O₂ pressure (Monk & Wadsö, 1975), temperature and the status of the inoculum used, these conditions can be controlled sufficiently within the laboratory. The reproducibility of thermograms of microbial growth as a technique of strain identification will allow the routine control of contamination, mutation and induced metabolic changes both during use and storage.

Tests for the identification of starter cultures are designed for species rather than strains and are based on morphology, differentiation by temperature and fermentative ability. Identification of strains requires extensive examination of their biochemical properties. However, with microbial thermograms, strains can be identified from one growth pattern when using carefully compounded medium.

Identification and comparison of a small number of bacteria from their thermograms is readily accomplished by visual appraisal although the height and placement of the characteristic features can vary even under strictly controlled conditions. Microbial thermograms reported in the literature are obtained with different calorimeters and growth conditions. While thermograms can be corrected for time constants arising from the calorimeter and sample volumes, detailed reporting of experimental procedure will be necessary before thermograms obtained in different laboratories can be compared. To allow interpretation of microbial thermograms, more information is required on the effect of medium composition, concomitant metabolism and inoculum properties as related to thermogram structure. With this information and the ability of a thermogram to describe microbial growth the technique might prove useful as an aid to classification of bacteria.

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VI

Microbial Calorimetry
as an
Analytical Method

The interpretation of heat production in relation to the underlying metabolic reactions of microbial growth has become an area of increasing interest and investigation. The method has developed from a specialised research aid towards being one of general analytical and industrial application. Recent developments and applications are briefly discussed.

The universal property of heat production associated with changes in the energy content of a system has analytical as well as thermodynamic considerations. The heat evolved or absorbed during a reaction is defined by the initial state of the reactants and the final state of the products, this is true for a reaction taking place in one step or the same reaction catalysed through numerous steps. The quantity of heat produced is additive and independent of the reaction mechanism and its rate. In biological reactions the environment is usually a solution subjected to neither volume nor pressure changes, which allows the thermodynamic quantity of enthalpy to be equated to the amount of heat evolved or absorbed. The analytical significance of measuring heat production is therefore its usefulness in quantitating a reaction. The design and operation of calorimeters and their application to biochemistry and biology has recently been reviewed¹.

A compound in a complex even turbid mixture can be quantitated by heat production if a specific reagent or catalyst such as an enzyme can effect its complete conversion². If the enthalpy change for the reaction is known, then the measured heat quantity can be related to the amount of compound reacted. However, it is usual to obtain a calibration value including the reaction and the calorimeter over the required substrate concentration. This procedure avoids corrections and calculations required when utilising tabulated thermochemical data. Enzyme activity can be measured by saturation with a specific substrate such that steady state kinetics can be maintained for sufficient time to allow the rate of heat production (heat effect) to be used as a measure of enzyme activity². By careful selection of buffer systems, reagents and coupled reactions the heat effects can be amplified.

One of the disadvantages of analytical calorimetry as applied to biological reactions is the difficulty in ensuring that the heat quantity observed is specific for the process being measured. Heats of dilution and mixing must be avoided or allowed for in carefully prepared blanks and standards. It is, however, the lack of specificity which has made calorimetry of significant application to complex biological processes including microbial metabolism. To interpret the heat

production which accompanies micro-biological metabolism, and apply the technique to practical problems, requires an understanding of its source and modification.

The simplest case of microbial metabolism to describe in terms of heat production is catabolism uncoupled from anabolism. Cells of many lactic acid bacteria, e.g. *Streptococci*, when washed, suspended in buffer and supplied with glucose will establish a constant rate of glycolysis. This will proceed anaerobically with essentially a homolactic fermentation and aerobically at the same rate with some modification of the products formed. In either case no work is done by the cells in increasing biomass or accumulating reserve material, but a small amount of energy is expended in the group translocation of glucose from the medium to the cell cytoplasm and in excluding some ions such as sodium and accumulating others such as potassium³. Classical equilibrium thermodynamics cannot be used to describe this open system, but the molar enthalpy change of glucose to products has been calculated for several bacteria⁴ and red blood cells^{5,6} and agrees with the measured heat production. The value indicates no net accumulation of ATP, which would considerably reduce the observed enthalpy change.

When placed in a nutritionally adequate medium with glucose as the energy supplying substrate the bacteria will grow, still essentially converting all the glucose to products, building cell mass from other medium compounds. For growth to occur the bacteria perform work in transporting and synthesising materials and organising the structure of the new biomass. The energy required to drive these reactions is made available by the enzymatically catalysed rearrangement of the substrate to products through the individual reactions of glycolysis. Some of the energy is conserved in discreet units of ADP phosphorylated to ATP, the amount determined by the metabolic pathway and partially utilised through coupled reactions during the transient existence of the cycling ATP.

The conservation of energy by the synthesis of biomass would be expected to reduce the amount of heat produced by catabolism. However, the contribution of the anabolic reactions to the measured heat production is negligible compared with catabolism of the energy supply substrate. Calculations on the conservation of energy in the chemical bonds formed in structuring the biomass agrees with the attempted calorimetric measurements⁴. It has also been estimated that only one tenth of the energy made available in ATP coupled reactions is utilised for synthetic work⁷.

The growth of a culture in a medium of adequate nutrition is expected to be described by a logarithmic increase in cell density, utilisation of substrate and formation of products and exhibit the conditions of balanced growth. The amount of heat liberated and its rate of production will then be described by the same first order rate constant as the other related parameters⁸. However, the thermogram of microbial growth is often profiled, the sensitivity and non specific nature of the calorimetric method showing deviations from balanced growth which may not be easily observed by changes in the rate of substrate degradation, utilisation of a different substrate or increase in cell density.

The correlation between the growth of a microbial culture and the resulting thermogram as being characteristic of an organism was first observed by Prat⁹. It has since been proposed that the thermograms of different bacteria could allow the identification of species and individual strains¹⁰. Many reproducible thermograms have been reported, however, some factors are recognised as greatly influencing the shape of microbial thermograms including instrumental, medium and inoculum properties. The changes in heat effect which occur in the calorimetric cell (thermogenesis) are modified by the thermal lags of the calorimeter. Rapid events may not be resolved and the thermogram will be distorted. This can be corrected electronically during recording or by processing a stored digitalised signal when the time constant of the calorimeter is known¹¹. Unless these corrections are made or determined not to be necessary, comparisons of thermograms from different calorimeters cannot be made.

If one component of a complex medium is replaced by another lot of similar material such as yeast extract or peptone, then a culture may give a different thermogram. The response of a culture to small changes in the growth medium can be a serious limitation for identification purposes; however, it has been successfully applied to optimising medium for growth of different cultures¹¹. The observed changes may not be a simple response to different or preferred energy substrates, but to limitation of growth factors which the culture may be required to synthesise during some stage of growth from degradation intermediates of the energy supplying substrate or other medium components.

Regulation of metabolism of one compound by varying concentrations of another may also be involved. The availability of oxygen to microaerophilic and facultative bacteria must also be controlled to obtain

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reproducible thermograms¹³. For identification purposes a completely defined medium has been found to be satisfactory, the thermograms are caused to be profiled and characteristic of different strains by addition of a mixture of carbohydrate energy sources. The concentrations are adjusted so that a number of them must be utilised before other conditions limit growth¹⁴. In Figure 1 are shown the thermograms of some representative strains of mesophilic lactic acid bacteria used in the preparation of dairy products, characterised in a defined medium.

Inoculum properties can cause alteration to a previously reproducible growth thermogram. The changes in the culture are solely related to the previous history of the inoculum which can be reverted or in some cases maintained. An example is *S. lactis* grown in a milk medium, energy source lactose, and used as an inoculum in a complex medium containing glucose⁶. Balanced growth on glucose was observed, but after several passages in the complex medium the culture grew with a profiled thermogram and acquired the ability to grow on other medium components. Events on the thermogram could be related to changes in the rate of glucose degradation and growth rate.

Analytical applications have developed parallel with increasing knowledge about microbial metabolism and the observed thermograms. Identification by calorimetry of microbial infections from blood cultures takes the time for growth of one culture¹⁵; however, the antibiotic used in treatment depends upon the strain's susceptibility. Calorimetry is used to examine the kinetics and mechanism of antibiotic action on microbial growth and for the determination of minimum inhibitory concentrations¹⁶. Combination of both identification and susceptibility testing concurrently would seem to be the next development. Bacteriuria has been enumerated by culturing urine samples in selected media for several hours prior to measuring the heat effect in a flow calorimeter, the method could be suitable for routine diagnostic use¹⁷. A similar approach without culturing has been made for monitoring potable water¹⁸ and examining microbial variation in sewage treatment (Tiefenbrunner, Personal communication). However, the available calorimetric designs require modification and multiple unit systems before such measurements can compete with existing techniques.

Microbial thermograms are useful in following the production of secondary metabolites. Staphylokinase accumulation in a medium mirrored the heat production by *Staphylococcus aureus* while the cell density continued to increase in an unrelated manner¹⁹. Calorimetry would appear to offer an advantage as an event timer either for the introduction of a precursor, inhibitor or in maximising the harvest of a labile product. Instrumental limitation has been a hindrance to its acceptance. Existing calorimeters require the ferment to be transported to a separately thermostated calorimetric vessel at a distance from the fermenter. A time delay of several minutes is not acceptable for monitoring most continuous cultures, while many batch cultures utilise particular or viscous media which cannot be conveniently pumped or representatively sampled by the small measuring volume of the calorimetric cell usually used. These problems have been

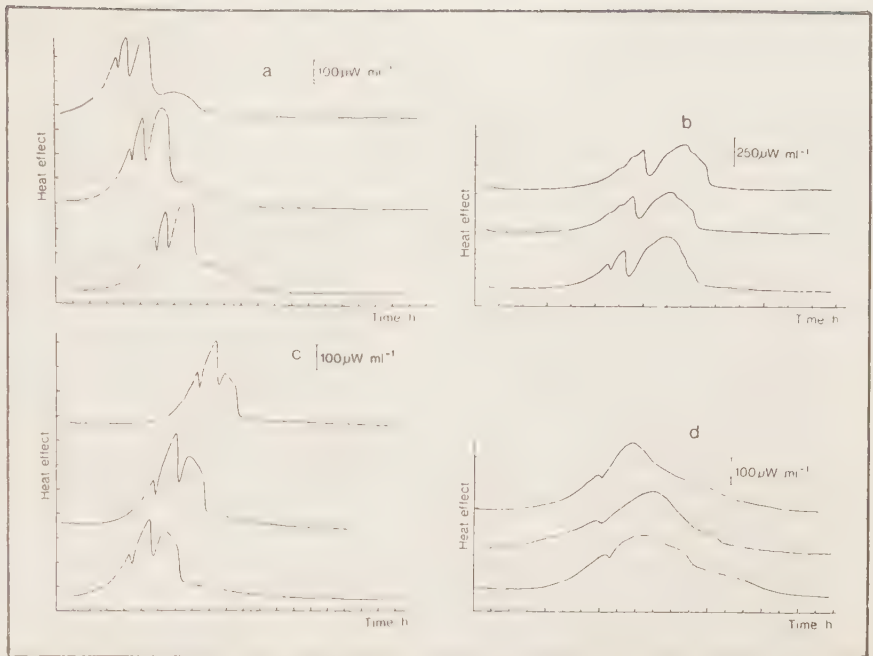


Figure 1. Growth of some mesophilic lactic acid bacteria in defined medium at 37°C. (a) *S. cremoris*; (b) *S. diacetylactis*; (c) *S. lactis*; (d) *Leuc. cremoris*. Three replicate thermograms are shown for each strain and displaced on the response axis for clarity. Inoculation made at zero hours (Fujita, et al., 1978).

successfully avoided by monitoring the whole ferment. Incremental temperature changes which occur when the temperature regulation is momentarily shut down can be utilised to construct a continual thermogram of the process²⁰.

Flow calorimetry is used to determine microbial contamination in water used in paper making²¹. The heat output is examined in relation to the inhibitory action of anti-slime agents, and the amount of inhibitor adjusted to effect bacteriostatic effects. The method gives faster results compared to plate counting and indicates a lower dose rate giving environmental and economic advantages.

Calorimetry has been recommended as a feasible technique for monitoring bacterial contamination of bulk prepared food-stuffs²¹.

The difficulty in interpreting the metabolic reactions giving rise to the thermograms of single cultures would indicate that application to mixed cultures would prove too complex. However, the total heat production associated with the complex symbiotic fermentation of cellulose in the rumen of the sheep could be related in a simple manner to the chemical processes occurring⁴. The symbiotic relationship of *S. thermophilus* and *L. bulgaricus* used in yogurt preparation has been examined by calorimetry²³. Using a small fermenter coupled to a flow calorimeter variation in the amount of inoculum and its composition, pH control and the action of cell free filtrates could be examined. In Figure 2 the effect of repeated subculture of the mixed culture on the stability of mixture in milk medium is compared with the thermograms of the individual strains. The inability of a strain of *S. cremoris* to grow in the presence of *S. lactis* even at ten times the rate of inoculation of the other, in a synthetic medium, is shown in Figure 3. In such applications it may not be necessary to understand, in detail, the microbial interactions for decisions to be made about selecting cultures for compatibility or a desired product. The ability of a thermogram to give a pictorial

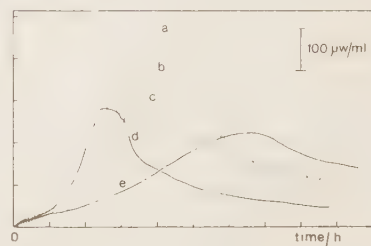


Figure 2. Thermograms (d) and (e) show the growth of *S. thermophilus* and *L. bulgaricus* in a milk medium at 37°C using a 1% (v:v) inoculum. Thermogram (a) was obtained with a 0.5% inoculum of each culture, (b) and (c) were obtained with 1% inoculum from (a) and (b) respectively.

record of changes in the resulting metabolism may prove sufficient.

The lack of reproducibility of thermograms due to different treatments of an inoculum is a useful technique for investigating inoculum

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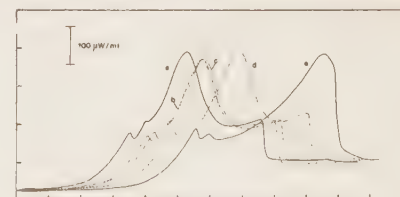


Figure 3. Thermograms of *S. lactis* and *S. cremoris* grown in a mixed culture. Defined medium containing 1% lactose, incubated at 37°C, was inoculated at the 1% level with an 8h culture of *S. lactis* (a) and *S. cremoris* (e). Mixed inoculum containing ratios of *S. lactis*: *S. cremoris* of 1:1, 1:2.5 and 1:10 generated the thermograms (b), (c) and (d) respectively. The decreased proportion of *S. lactis* in the mixed inoculum caused an increase in lag time, but *S. lactis* dominated all the fermentations.

Microbial Calorimetry as an Analytical Method

Continued from page 5

properties. In Figure 4 are shown thermograms obtained with an inoculum of *S. lactis* stored at -22°C for different times. A defined medium containing 1% lactose was used. Progressive changes in the thermograms indicate metabolic alteration to the inoculum during storage. Initial observations using inoculum stored at -22°C have shown great variability between strains in both metabolic changes and the ability to recover on repeated subculture. If the observed effects are due to damage of transport systems or metabolic pathways remains to be determined. The storage properties of yeast have also been examined.²⁴

Of ecological importance are the calorimetric studies to assess the action of residual herbicides²⁵ and acid fall-out on the overall metabolism of the mixed microbial populations in soils²⁶.

Many biological materials have been presented to calorimeters and heat effects with a variety of patterns have been observed; however, the ease of obtaining information is often confounded by the inability to interpret the results. Where, after consideration, calorimetry has been selected as a method of solving a specific problem and the experiment designed to account for heat production from

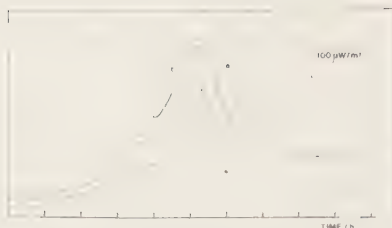


Figure 4. Effect of storage time on the fermentation properties of frozen inoculum of *S. lactis*. The inoculum was stored at -22°C and thawed at room temperature before use. Thermogram (a) shows growth in defined medium containing 1% lactose and using unfrozen inoculum. Inoculum frozen for 3 days gave (b) and for 17 days (c). The main peak (growth on lactose) occurred at approximately the same time, the ability to degrade other compounds varied with storage time. Fermentation (c) when used as an inoculum demonstrated restored metabolic ability as for (a).

artefact reactions, then the method can be superior to others.

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VII

Aerobic Growth Thermograms of *Streptococcus lactis* Obtained with a Complex Medium Containing Glucose

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With different culturing methods both simple and complex thermograms were obtained with *Streptococcus lactis* grown aerobically in a complex medium containing growth-limiting concentrations of glucose. The thermogram profiles have been interpreted in relation to growth rate, glucose degradation, and molar growth yields calculated for different time intervals during growth.

The record of heat production during microbial growth, a thermogram, may be characteristic of an organism when recorded under controlled growth conditions (2, 4, 7, 14). Only a few investigations have been made to determine the cause of heat profiles in relation to the underlying metabolic reactions (1, 10, 12); however, this information will be important for the interpretation of microbial thermograms and their application to practical problems.

When grown in a nutritionally adequate medium, a culture will be expected to grow logarithmically, and the increase in biomass, degradation of the energy-supplying substrate, and formation of products of reaction can be described by the same rate constant. Under these conditions the increase in heat production will also be described by the same rate constant. This observation has been reported for organisms as different as *Streptococcus faecalis* growing with limiting concentrations of glucose (6) and *Saccharomyces cerevisiae* degrading several carbohydrates (11). Changes in the rate of heat production during growth will therefore be expected to reflect the catabolic and synthetic abilities of a culture in response to its growth environment and will be related to changes in growth rate, degradation of energy-supplying substrates, and formation of reaction products.

In developing calorimetric methods for the identification of lactic acid bacteria (7), variable thermograms were obtained for *Streptococcus lactis* previously maintained in milk medium and then passaged on a complex medium containing glucose. The thermograms obtained using inoculum prepared after one passage in complex medium were simple, giving a single logarithmic increase in heat production. After several passages the inoculum then gave a reproducible, but complex, thermogram.

In this report inocula prepared to give both types of thermograms have been used in com-

plex medium containing growth-limiting concentrations of glucose. Growth rate, glucose degradation, and molar growth yield (MGY) calculated during different stages of growth have been used to interpret the thermogram.

MATERIALS AND METHODS

Bacterial strain. The culture used was *S. lactis* 38 obtained from the Swedish Dairy Association, Malmö, Sweden.

Media. Low-heat skim milk medium (10%, wt/vol; Scanmilk, Malmö, Sweden) was used for daily maintenance of the culture. The basal complex medium used for preparing inoculum and for growth experiments contained, per liter: tryptone, 5 g; nutrient broth, 8 g; yeast extract, 5 g, (Difco Laboratories, Detroit, Mich.); ascorbic acid, 0.5 g; KH_2PO_4 , 5 g; K_2HPO_4 , 5 g; and 1 mM MgSO_4 . When used for daily maintenance, the medium contained, per liter, 10 g of glucose and 8 g of β -disodium glycerophosphate to increase buffer capacity. The media were adjusted to pH 6.5 before autoclaving at 121°C for 10 min.

Preparation of inoculum and growth experiments. The inoculum for growth experiments was prepared in batch culture from 700 ml of the basal complex medium, containing 7 g of glucose sterilized as a 20% (wt/vol) solution and added separately. The medium was contained in a 1-liter Biotec fermentor (LKB-Produkter, Bromma, Sweden) maintained at 37°C and magnetically stirred. The pH was continually adjusted to 6.5 by the addition of 5 M NaOH, using a Radiometer pH meter (TTT1C, Radiometer, Copenhagen, Denmark) coupled to a metering pump giving on-off control. The medium was pumped at 60 ml/h through a flow calorimeter maintained at 37°C and returned to the fermentor. The microaerophilic property of the culture, stirring the fermentation, and the fast flow rate would ensure that aerobic conditions were maintained in both the fermentor and the calorimetric cell.

To obtain an inoculum giving growth with a logarithmic increase in heat production, cells were grown for one passage in basal complex medium containing glucose. Medium in the fermentor was inoculated with 7 ml of a 16-h culture grown in milk medium, and at

the end of growth 7 ml of this culture was used, within 1 h, to inoculate 700 ml of complex medium containing approximately 6 mM glucose. Other samples were stored at -22°C for 1 or 2 days before being used as an inoculum. The thermograms were repeated several times with and without the addition of glucose. Analysis of bacterial growth and glucose degradation were made in conjunction with the thermograms.

Growth thermograms were also obtained by using an inoculum prepared from a culture grown in the complex medium for 10 passages. Seven milliliters of this 16-h culture was then used to inoculate basal complex medium containing glucose, placed in the fermentor. To obtain a reproducible supply of inoculum, 200 ml of the culture was harvested at the end of growth, centrifuged, washed, and suspended in sterile phosphate buffer (5 g of KH_2PO_4 and 5 g of K_2HPO_4 in 1 liter at pH 6.5). Seven-milliliter samples were dispensed into glass tubes and stored at -22°C . Thermograms were obtained with this inoculum by thawing at room temperature and then used to inoculate complex medium containing glucose at different concentrations from 2 to 11 mM. Thermograms of growth in the medium without added glucose were obtained in four experiments. In two experiments where the glucose concentrations were approximately 6 and 8 mM, growth rate and glucose degradation were measured in conjunction with the thermograms.

Thermograms of fermentations can be affected by the storage time of the frozen inoculum. The random selection of the glucose concentrations added to the medium and comparison between frozen and unfrozen inocula were used as a control of inoculum stability.

Analytical methods. The flow calorimeter used was of a design similar to that reported earlier (8). The steel flow cell was replaced by a Teflon tube of 0.8-ml volume, embedded in Woods metal. The base line stability was $6\ \mu\text{W}/24\ \text{h}$, and the half-time response of the calorimeter was 2.2 min. The growth rate of the cultures was slow enough not to require dynamic correction of the thermograms.

The Teflon cell and calorimeter flow lines were sterilized with 1 M NaOH, followed by sterile distilled water and growth medium. Cell density was measured by centrifuging samples, suspending the cells in 0.1 M phosphate buffer, and measuring the optical density at 650 nm with a Shimadzu SPQ50 spectrophotometer. The cell dry weight was determined from a calibration curve. One-milliliter samples for glucose analysis were dispensed into 2 ml of 8% (vol/vol) perchloric acid and centrifuged, and glucose in the supernatant was determined by the GOD-Perid method (Boehringer, Mannheim, Germany).

RESULTS

Thermograms (Fig. 1) were obtained with inoculum after one passage in complex medium, using unfrozen inoculum A and frozen inoculum B; both cultures contained approximately 6 mM glucose. An increase in lag time occurred in B, compared to A, a consequence of loss in viability of the frozen inoculum. The area enclosed by both thermograms and the doubling time for the

increase in heat effect were essentially the same. During the recording of thermogram B, growth rate and glucose degradation were measured and compared with the increase in heat effect (Fig. 2). The respective doubling times were 40, 41, and 41 min, and the MGY was 54.6 g/mol of

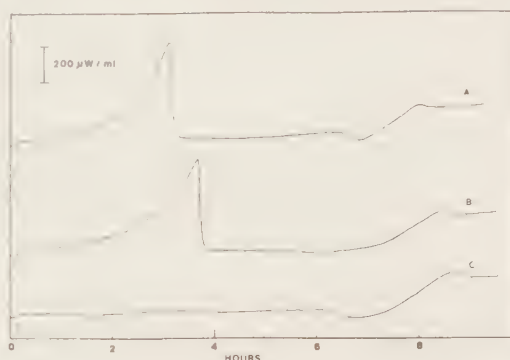


FIG. 1. Thermograms of *S. lactis* grown at 37°C on complex medium containing approximately 6 mM glucose (A and B) or without glucose (C). The inoculum was prepared from a culture passaged once in complex medium after maintenance in 10% (wt/vol) skim milk medium. The inoculum giving thermogram A was used 1 h after harvesting, and that giving thermogram B was used after storage at -22°C for 48 h.

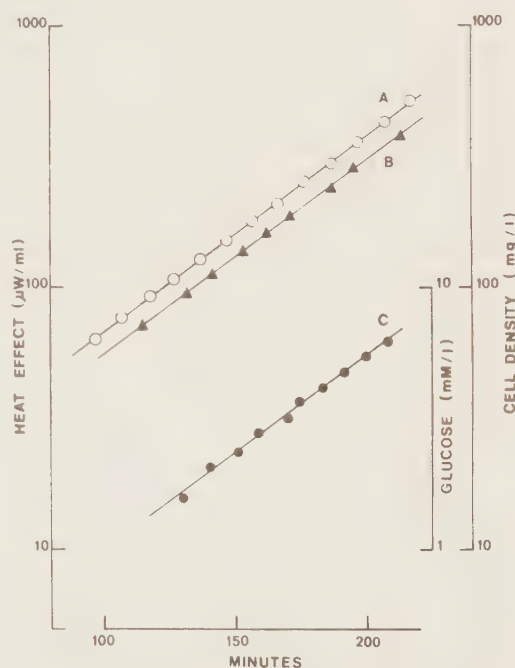


FIG. 2. Analysis of thermogram B of Fig. 1. Symbols: Logarithmic increase in heat effect (○); cell density (▲); amount of glucose degraded by the culture (●).

glucose (Table 1). The area under thermogram B, the heat produced, gave an enthalpy change of -219 kJ/mol of glucose and was not corrected for buffer effects.

In both thermograms the heat effect increased logarithmically, accompanied by utilization of glucose in the medium and an increase in cell biomass. When glucose degradation was complete, the heat effect decreased rapidly to a low endogenous level, and no further increase in cell density occurred until 3 h later, when the heat effect was seen to increase.

Thermogram C was recorded in the absence of glucose. The second increase in heat effect appeared in all the thermograms at the same time independent of previous growth on glucose. This heat effect increased slowly and, after reaching a maximum value, declined slightly to a steady-state level, which continued for several hours. The increase in heat effect was associated with growth of the culture. In thermogram C, no significant growth occurred until the appearance of this peak.

The thermograms (Fig. 3) were recorded during growth of *S. lactis* on the complex medium, using inoculum prepared after the culture had been passaged 10 times in maintenance medium. Thermogram A was obtained without glucose added to the medium, and thermogram B was obtained when the medium contained 7.59 mM glucose, similar to thermograms recorded for cultures containing different glucose concentrations. Thermogram A was typical of similar thermograms obtained for growth without glucose. The lag time, increase in heat effect, and maximum heat effect remained the same and were independent of storage time of the frozen inoc-

ulum. The increase in heat effect was accompanied by growth; further growth did not occur after resolution of the first peak in the thermogram. The medium component which supported growth in the absence of glucose was not determined. Thermogram B showed two main peaks enclosing two small but reproducible peaks. This thermogram was representative of similar thermograms obtained with other glucose concentrations; however, the height of the second main

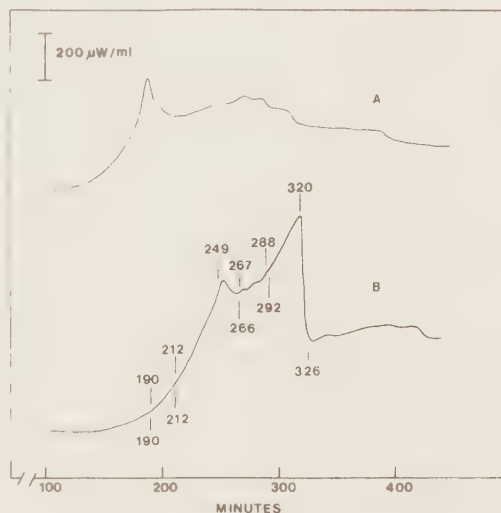


FIG. 3. Thermograms of *S. lactis* grown at 37°C on complex medium without glucose (A) or containing 7.6 mM glucose (B). The culture had been passaged 10 times in maintenance medium before use as an inoculum. Times for changes in rate of glucose degradation are marked above and changes in growth rate below the thermogram (B).

TABLE 1. MGYs and mean doubling times for growth rate, glucose degradation, and heat effect

Time interval (min)	Glucose degraded (μ mol/ml)	Yield (μ g [dry wt]/ml)	Y(glucose) (g [dry wt]/mol) ^a	Coefficient of determination	Mean doubling time (min)		
					Growth rate	Glucose degradation	Heat effect
0-190	0	16			12		16
212-249	1.13	49	45.8	0.989	19	14	18, 26 ^b
250-265	0.29	45	166	0.986	19	47	
267-288	1.96	100	51.6	0.993	25	17	
293-320	3.73	185	48.3	0.991	34	24	
0-330 ^c	7.59	540	71.1				
0-200 ^d	0	302					17
0-330 ^e	7.59	238	31.4				
0-210 ^f	6.37	340	54.6	0.986	40	41	41

^a Calculated from a linear regression between cell yield and glucose degraded for the time intervals shown. The coefficient of determination is shown for each regression.

^b A change in rate of heat effect occurred at 216 min without a corresponding change in either growth rate or glucose degradation.

^c Overall MGY for culture, Fig. 3B.

^d Growth without glucose, Fig. 3A.

^e Corrected MGY, Fig. 3B.

^f Data for culture, Fig. 1B.

peak when measured from peak to trough was linearly related to the concentration of glucose added to the medium. With the glucose concentrations studied, the maximum heat effect of the first peak remained constant.

The results of analysis of the cultures giving the thermograms (Fig. 3) are shown (Fig. 4). Times of changes in the rate of glucose degradation and increase in cell density observed in Fig. 4C and D and are indicated above and below the thermogram (Fig. 3B). The logarithmic increase in heat effect until the appearance of the first peak in the thermograms (Fig. 3) are plotted for growth without and with glucose (Fig. 4A and B). In the absence of glucose the increase could be described by one logarithmic rate until the first peak was resolved, whereas in the presence of glucose the increase in heat effect was at three rates. Similar results were found in repeated experiments. The logarithmic degradation of glucose and increase in cell density are shown in Fig. 4C and D, respectively. Although the culture containing glucose began growing at about 140 min after inoculation, glucose degradation did not begin until 190 min, and then its degradation was best described by five logarithmic rates until its utilization was completed at 320 min. When glucose degradation began at 190 min, growth stopped, and a reduced rate of heat production was observed (Fig. 4B). No further increase in cell density occurred until 212 min, at which time the rate of glucose degradation decreased. However, no change in heat production was observed until 10 min later. When the culture resumed growing at 212 min, its growth was described by three logarithmic rates until

the glucose degradation was complete and growth ceased. With another culture of lower glucose concentration in the medium, both the growth rate and glucose degradation followed a similar pattern. However, growth stopped for only 10 min when glucose degradation began. It can be seen (Fig. 4C and D) that at no time during growth did the logarithmic rate of glucose degradation have the same value as the growth rate. Except for the period between 249 and 267 min, the rate of glucose degradation was greater than the corresponding growth rate. Changes in the rate of glucose degradation occurred at the same time as changes in growth rate at 212, 266, and 292 min. At 249 min glucose degradation decreased without a corresponding change in growth rate.

Changes in the rate of heat effect observed from the thermogram (Fig. 3B) show a good correlation with changes in the rate of glucose degradation marked above and changes in growth rate marked below the thermogram. The section of the thermogram between 190 and 212 min shows an increase in heat effect, although no growth was observed to occur. However, glucose degradation began at 190 min and increased rapidly until 212 min, accounting for the continued increase in heat effect.

Only a small increase in cell density of 16 $\mu\text{g}/\text{ml}$ was measured from 140 to 190 min, and 0.2 $\mu\text{mol}/\text{ml}$ of glucose was degraded between 190 and 212 min. These values are subject to a large error; however, the heat production values measured during the same time interval are more reliable because of the calorimeter sensitivity.

From Fig. 4C and D an MGY has been calculated for each time interval which showed a different logarithmic rate of glucose degradation. The slope of a linear regression between cell yield and glucose degraded gave the MGY; the coefficient of determination was calculated for each regression (Table 1). An MGY was calculated for the overall fermentation and corrected for growth in the absence of glucose and for the culture showing simple logarithmic growth. The mean doubling time for growth rate, glucose degradation, and, where possible, for the increase in heat effect has been calculated (Table 1).

DISCUSSION

The heat produced by a growing culture has three main components: the enthalpy change associated with catabolism of the energy-supplying substrate, the heat of ionization and dilution of the degradation products, and the enthalpy change of growth. In a phosphate buffer the

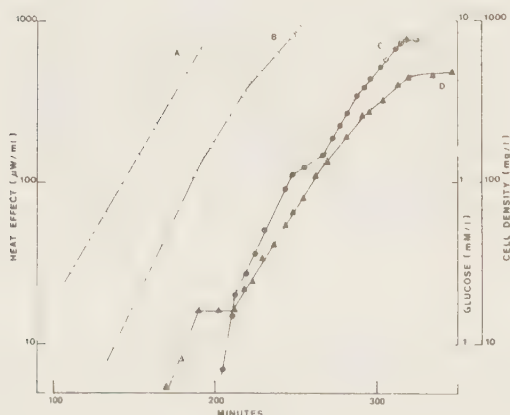


FIG. 4. Analysis of thermograms from Fig. 3. Logarithmic increase in heat effect without glucose (A) and with glucose (B). For the culture containing glucose, the logarithmic increase in cell density (▲) and the amount of glucose degraded by the culture (●) are shown.

product-associated heats are small, and in a complex medium the contribution from the anabolic processes can be calculated as being very small (5). A thermogram records the heat effect (the rate of heat production) as growth progresses. The rate of increase in heat effect will be primarily related to the rate of increase in cell mass, whereas its magnitude will depend upon the cell density, rate of catabolism, and the enthalpy change associated with catabolism. It follows that a decrease in heat effect during growth could be attributed to a decrease in the rate of catabolism, a change in the proportions or types of degradation products, or utilization of different energy sources.

The expected thermograms (Fig. 1A and B) were obtained for a culture growing in a nutritionally adequate medium where growth had been limited by energy source. The growth could be described by a similar rate constant for increase in cell density, degradation of glucose, and increase in heat effect (Fig. 2). Growth of a culture showing these properties has been described as balanced (13). Although the shape of this thermogram was similar to that obtained for *S. lactis* grown anaerobically (5), the enthalpy change of -219 kJ/mol was twice as large, assuming glucose to be the only energy source. However, a high enthalpy change is not unexpected for aerobic growth of microaerophiles, since the heat produced and the shape of the thermogram can be significantly different (9). The high MGY, 54.6, indicates that glucose may not have been the only energy-supplying substrate, even applying the high aerobic coupling efficiency of 20 g/mol of ATP reported for *S. diacetylactis* and *S. cremoris* (3).

The two different thermograms obtained with *S. lactis* grown in complex medium containing limiting amounts of glucose (Fig. 1B and 3B) appear to be solely related to the method of preparing the inoculum. Why the growth of the culture changed between 1 and 10 passages in complex medium is not known; however, the result of the metabolic alteration can be seen from the thermogram.

The coefficients of determination (Table 1) show that a good linear correlation exists between the increase in cell density and glucose degraded during each time interval in which glucose is degraded at a different logarithmic rate (Fig. 4C). A linear relationship also exists between the doubling times for growth rate and glucose degradation if the time interval of 250 to 265 min is disregarded. The results demonstrate a dependent relationship between the growth rate and the rate of glucose degradation until glucose is used up and growth stops. However, unlike the culture giving the results shown in

Fig. 2, the doubling time for glucose degradation is faster than the corresponding growth rate for each time interval, except 250 to 265 min. These observations lead to one possible conclusion, that the efficiency of coupling of glucose degradation to the increase in cell biomass is constant during the growth of the culture showing the thermogram (Fig. 3B) but lower than the efficiency of coupling when balanced growth is observed (Fig. 1B).

The MGY calculated for each glucose degradation rate (Table 1) must be considered an apparent value, because the contribution to cell yield by energy-supplying substrates other than glucose are included in the linear regression method of calculation. Growth by this culture in the absence of glucose is large, 302 μ g/ml. When this value is subtracted from growth with glucose a corrected MGY of 31.4 is obtained. If this value is taken as the efficiency of coupling of glucose degradation during growth, then an estimate of the contribution of other energy sources to the increase in cell mass can be calculated for each section of the growth curve. For the interval 250 to 265 min, 80% of the measured increases in cell mass would be attributed to other energy sources, whereas the other intervals would range from 28 to 39%.

The doubling time for growth increased from about 12 to 34 min as growth progressed, approaching the 41-min doubling time of the culture showing only one growth rate constant. The doubling time for the heat effect for growth without glucose (Fig. 3A) was 17 min, similar to the value of 16 min for growth in the medium containing glucose but before glucose degradation began. Growth appears to have begun on the unknown compound which could support rapid growth, but when glucose degradation began its rate of degradation decreased.

The changes in heat effect shown by the thermogram in Fig. 3B can be correlated with changes in rate of glucose catabolism. The rate of glucose degradation, however, is a consequence of the growth rate; hence, the observed changes in growth rate must be coupled to the utilization of other medium components.

Growth of some bacteria as represented by *S. lactis* are dependent upon the fastidious requirement for nearly all the necessary amino acids and vitamins, using the degradation of the substrate to supply the energy for growth. Other bacteria such as *Escherichia coli* can grow successfully and logarithmically in a simple medium containing glucose, from which they furnish their requirements for catabolites, growth factors, and energy (1). Within this range of behavior may be found a large number of thermograms reflecting energetic changes during growth, the

summed inefficiency of metabolic coupling at any time being observed as heat production.

The suggested characteristic nature of thermograms of individual bacterial strains, as applied to their identification, may be the result of response to the medium by their constitutive or induced metabolic ability. The addition or deletion of selected medium components would then result in a state of balanced growth.

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VIII

Thermograms of *Streptococcus thermophilus* and *Lactobacillus bulgaricus* in single and mixed culture in milk medium

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SUMMARY. Microbial thermograms have been used to investigate the growth of *Streptococcus thermophilus* and *Lactobacillus bulgaricus* in single and mixed culture in milk medium. Growth has been observed with different ratios of the strains in the inoculum and, at one ratio, the effect of the amount of inoculum. The effect of serial sub-culture both with and without pH control has been observed, and stimulation of *Str. thermophilus* by cell-free filtrates of *L. bulgaricus* has been examined. The development of the technique is discussed in terms of the application of microbial thermograms in selecting cultures for incorporation into mixtures and evaluating their performance.

Many dairy products are prepared using mixed starter cultures, either added as an inoculum of well-defined composition, or by repeated subculture of originally mixed strains. For many mixtures only a few subcultures are required for one strain to become dominant, due to different growth rates, temperature optimum and possibly inhibition by metabolites (Lawrence, Thomas & Terzaghi, 1976). However, the mixture of *Streptococcus thermophilus* and *Lactobacillus bulgaricus* grows symbiotically with increased growth and acid production (Moon & Reinbold, 1976). The metabolites involved in stimulating the growth of both bacteria are still being investigated (Shankar & Davies, 1977).

The associative growth of these 2 bacteria has been studied under batch conditions (Moon & Reinbold, 1976; Accolas *et al.* 1977) and to establish conditions for the continuous fermentation of milk to yoghurt (Lelieveld, 1976; Driessen, Ubbels & Stadhouders, 1977). The methods used to investigate the growth of *Str. thermophilus* and *L. bulgaricus* in mixed culture have been the rate and amount of acid production and the increase in cell numbers by differential plate counts.

A technique that has not been applied which can give a continuous record of growth under batch and steady-state conditions is calorimetry. The heat production associated with microbial growth can give thermochemical information about microbial metabolism (Forrest, 1972), while the heat effect recorded during growth (a thermogram) may be characteristic of a particular strain. Profiled thermograms characteristic of individual strains of lactic acid bacteria have been obtained with a defined medium containing limiting concentrations of several carbohydrates, while with a complex medium containing excess of an energy source the thermograms lacked significant identifying features (Fujita, Monk & Wadsö, 1978). Simple thermograms are also associated with the growth of mixed cultures in natural environments such

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as rumen digesta (Forrest, 1972), soil (Mortensen, Norèn & Wadsö, 1973), and polluted water (Tiefenbrunner, 1976). The complex microbial populations, accompanied by simple thermograms, have discouraged attempts to characterize interactions between strains and contributions to the heat effect by individual strains (Schaarschmidt & Lamprecht, 1976).

This report deals with an investigation of the application of microbial thermograms to evaluating the performance of mixed cultures. The growth of *Str. thermophilus* and *L. bulgaricus* together in a milk medium was examined in relation to the composition and amount of the inoculum, stability of the mixed cultures on repeated subculture, growth at controlled pH and the addition of cell-free filtrates to the milk medium.

The calorimetric observations were evaluated and compared with the expected microbiological properties of the mixture and the results used to assess the proposed application of the technique to the selection of strains for incorporation into mixed cultures and determination of their stability during use.

MATERIALS AND METHODS

Microorganisms

The bacteria used were *Str. thermophilus* CNRZ 302 and *L. bulgaricus* CNRZ 86 supplied from the culture collection of the Swedish Dairy Association, Malmö, Sweden.

Stock cultures and media

The cultures were maintained by daily sub-culture into 10% (w/v) reconstituted low heat skim-milk (Scanmilk, Sweden) sterilized by autoclaving at 121 °C for 10 min. Inoculum for growth experiments was taken from an 8-h culture grown in 10% (w/v) milk medium. For calorimetric measurements, 5% (w/v) milk medium was used and the incubation temperature was always 37 °C.

Inoculum

All inoculations, unless otherwise stated, were made at the 1% (v/v) level, both for single and mixed culture. The relationship between the 2 strains in the mixed fermentations is reported as the ratio of the number of *L. bulgaricus* to *Str. thermophilus*. A 1% inoculum of *Str. thermophilus* gave approximately 1.2×10^6 cells/ml and *L. bulgaricus* 4.5×10^6 cells/ml, determined from plate counts using plate count agar DIFCO.

Calorimetry

A flow calorimeter of a similar design to that reported earlier was used (Monk & Wadsö, 1968), but the steel flow cell was replaced by a Teflon tube (which allows easy passage of air bubbles) of 0.8 ml embedded in Woods metal. The calorimeter had a baseline stability of 6 μ W/24 h and a half-time response of 2.2 min. The changes in heat effect were so slow that thermograms did not require dynamic correction. The calorimetric cell and flow lines were sterilized with 1 M-NaOH, followed by sterile water and then medium. The calorimeter at 37 °C was connected to a 1-l Biotec fermenter (LKB, Bromma, Sweden) containing 700 ml sterile milk medium, magnetically stirred and maintained at 37 °C. The medium was pumped at 60 ml/h through

the calorimeter and returned to the fermenter. In experiments where the pH was maintained at 6.5, 5 M-NaOH was added to the fermenter by a PERPEX peristaltic pump (LKB, Bromma, Sweden) controlled by a Radiometer pH meter (TTT1C, Radiometer, Copenhagen, Denmark). Due to stirring, the use of Teflon flow lines, the fast flow-rate and the microaerophilic properties of the cultures, all the fermentations can be considered aerobic.

Calorimetric measurements

Inoculum effect. To study the effect of different proportions of the 2 strains in the inoculum on the growth thermogram, different volumes of 8-h cultures were mixed together and contained *L. bulgaricus* and *Str. thermophilus* in ratios of 0.6:1, 1.5:1, 3.8:1 and 49:1. The mixed inocula were used at the 1% level and the thermograms obtained were compared with those of the single cultures.

To assess the contribution of the quantity of inoculum to the growth of the mixed culture, milk medium was inoculated with 0.25, 0.5, 1.0 and 2% (v/v) of a mixed inoculum containing equal volumes of 8-h cultures of each strain. This gave a cell density in the inoculated milk medium ranging from $1-9 \times 10^6$ cells/ml of *L. bulgaricus* and $0.3-2.4 \times 10^6$ cells/ml of *Str. thermophilus*, while maintaining a constant ratio between *L. bulgaricus* and *Str. thermophilus* of 3.8:1.

To examine the effect of repeated subculture the following procedure was followed. Milk medium was inoculated with 1% (v/v) of a mixed inoculum of 8-h cultures containing *L. bulgaricus* to *Str. thermophilus* in a ratio of 3.8:1 and the thermogram recorded. After 9 h a portion of the fermentation was removed and used to inoculate another batch of sterile milk medium at the 1% (v/v) level, and the growth thermogram was recorded. A third thermogram was obtained using a 9-h inoculum from the second fermentation. The thermograms obtained by serial inoculation were compared with those of the single strains and the other inoculation experiments.

Growth of *Str. thermophilus* in milk medium supplemented with cell-free filtrates

Milk medium 5% (w/v) was supplemented with 20% (v/v) cell-free filtrates of milk medium in which *L. bulgaricus* or *Str. thermophilus* had grown for 8 h, and then inoculated with 1% (v/v) of an 8-h culture of *Str. thermophilus*. The thermograms obtained were compared with *Str. thermophilus* grown without added supplements. The cell-free filtrates were prepared from 200 ml of 8-h cultures grown in 5% (w/v) skim-milk medium. The coagulated products were adjusted to pH 4.5 with 5 M-HCl and after standing at room temperature for 30 min the precipitate was removed by filtration. The filtrate was adjusted to pH 6.5 with 5 M-NaOH and then centrifuged. The supernatant was sterilized by filtration through a Millipore assembly (0.45 μ m pore size) before addition to sterile milk medium. The initial concentration of the milk medium was adjusted to give a 5% (w/v) solution after dilution with the cell-free filtrate.

The effect of pH on growth of the cultures

Both single strains were grown in 5% (w/v) milk medium at pH 6.5, which was maintained by the addition of 5 M-NaOH to the fermenter. The thermograms obtained were compared with those grown without pH control. Growth of a mixture of the 2 strains was examined in a similar way using a 1% inoculum containing a 3.8:1 ratio of *L. bulgaricus* to *Str. thermophilus*. To examine the mixed fermentation on

repeated subculture, 3 consecutive sub-cultures were made using a 1% inoculum after growth of the cultures at pH 6.5.

RESULTS

Fig. 1(A) shows the thermograms obtained when different amounts of mixed inoculum were used containing *L. bulgaricus* and *Str. thermophilus* in a constant ratio of 3.8:1. The thermograms (*a*, *b*, *c* and *d*) correspond to inoculation concentrations of 2, 1, 0.5 and 0.25% (v/v) respectively. The only characteristic feature of the thermograms was the appearance of a shoulder on the descending side of the otherwise bell-shaped heat effect curve. The maximum heat effect and the heat produced by the fermentations were essentially independent of the amount of inoculum, while the time taken to reach the maximum heat effect increased with decreasing amounts of inoculum (Table 1).

For the thermograms (*a*, *b*, *c* and *d*), Fig. 1(B), the ratio of *L. bulgaricus* to *Str. thermophilus* in a 1% (v/v) inoculum was 0.6:1, 1.5:1, 3.8:1 and 49:1 respectively. Thermograms (*e*) and (*f*) were obtained with the individual cultures of *Str. thermophilus* and *L. bulgaricus* respectively. *L. bulgaricus* produced 48% more heat during 9 h than *Str. thermophilus* in single culture, while *Str. thermophilus* gave a slightly higher maximum heat effect several hours before *L. bulgaricus*.

For the mixed cultures the heat produced was essentially constant and independent of the composition of the inoculum and equal to 75% of the sum of the heat produced by the single cultures. The maximum heat effect occurred when the ratio of *L. bulgaricus* to *Str. thermophilus* was 3.8:1, and equalled 88% of the sum of the maximum heat effects for the single cultures. *Str. thermophilus* reached a maximum heat effect at 2.8 h and *L. bulgaricus* at 6.1 h after inoculation, while for the mixed cultures the time increased with increasing numbers of *L. bulgaricus* and decreasing numbers of *Str. thermophilus* in the inoculum. The results are summarized in Table 1.

Thermogram (*a*), Fig. 2(B), was obtained using a 1% (v/v) mixed inoculum containing *L. bulgaricus* and *Str. thermophilus* in a ratio of 3.8:1. Thermograms (*b*) and (*c*) were obtained by using inocula taken from the fermentation giving the thermograms (*a*) and (*b*) respectively. The heat production for the first fermentation was equivalent to 78% of the combined heat production for the single cultures given by *Str. thermophilus* and *L. bulgaricus*, shown by thermograms (*d*) and (*e*). The second fermentation gave a similar result, but at the third fermentation the heat production decreased to 67%. The maximum heat effect of the first mixed culture was equivalent to 90% of the sum of values for the 2 individual cultures, but decreased by approximately 10% at each subsequent subculture. The time required to reach the maximum heat effect was reduced at each subculture. The results are summarized in Table 1.

The thermograms in Fig. 2(A) show the growth of *Str. thermophilus* in medium supplemented with a 20% (v/v), 8 h, cell-free filtrate of *L. bulgaricus* (*a*), without supplement (*b*) and with a 20% (v/v), 8 h cell-free filtrate of *Str. thermophilus* (*c*). Filtrate from *L. bulgaricus* stimulated the heat effect and heat produced by about 30% while the addition of *Str. thermophilus* filtrate depressed these values by 10% (Table 2). The maximum heat effect was reached at the same time for all the cultures.

The logarithmic increase in heat effect for each of the thermograms in Fig. 2(B) is plotted in Fig. 3, where *Str. thermophilus* and *L. bulgaricus* are shown by (*d*) and (*e*),

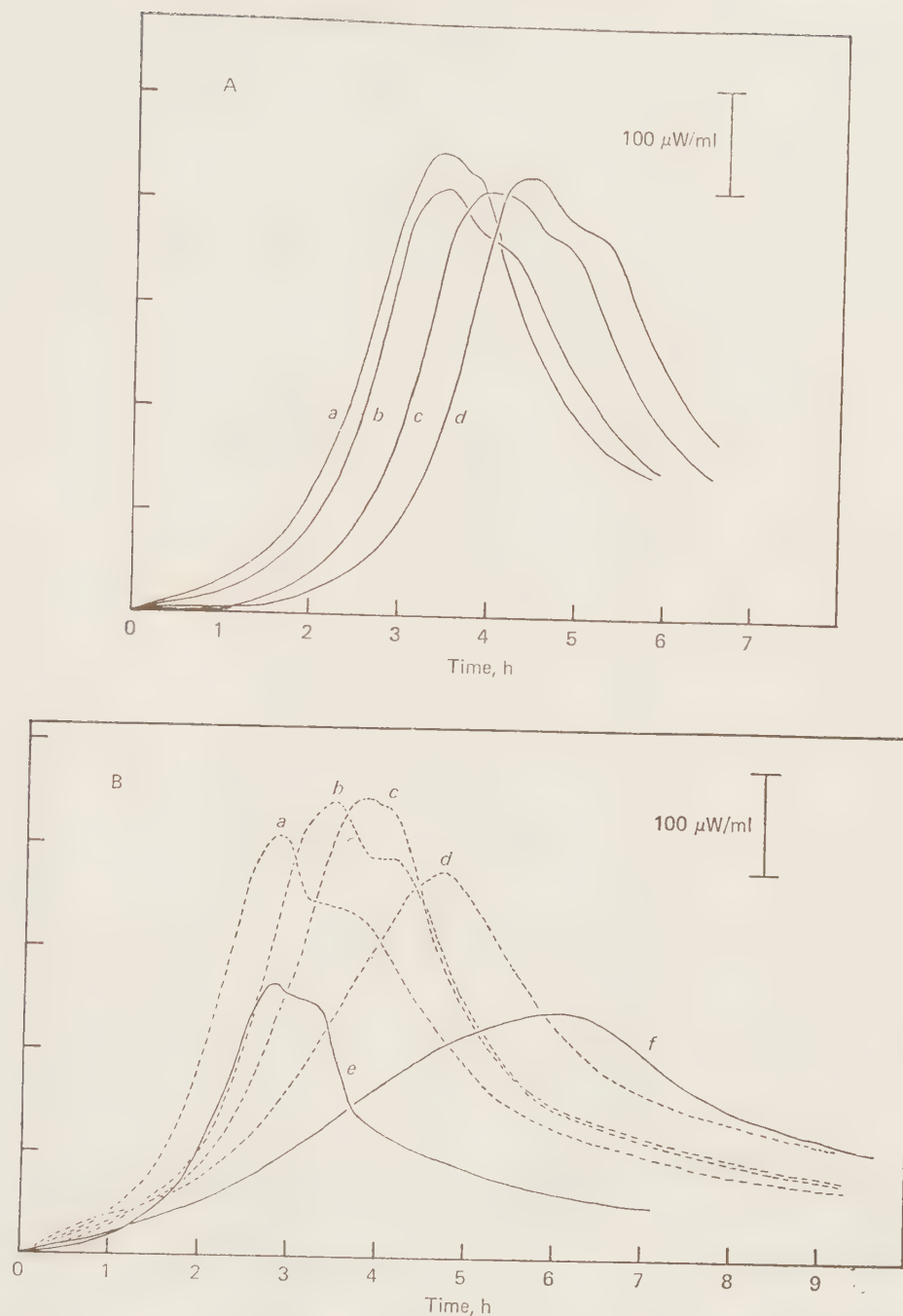


Fig. 1. (A) The effect of the amount of inoculum containing *Lactobacillus bulgaricus* and *Streptococcus thermophilus* in a ratio of 3.8:1. Thermograms a, b, c and d were obtained with 2.0, 1.0, 0.5 and 0.25% (v/v) respectively of mixed inoculum. (B) The effect of the ratio of *L. bulgaricus* to *Str. thermophilus* contained in a 1% (v/v) inoculum. Thermograms a, b, c and d were obtained with ratios of 0.6:1; 1.5:1; 3.8:1 and 49:1 respectively. Single cultures of *Str. thermophilus* and *L. bulgaricus* are shown by thermograms e and f.

Table 1. *The effects of amount of inoculum, ratio of Lactobacillus bulgaricus: Streptococcus thermophilus and serial inoculation on the heat production and the maximum heat effect*

	Amount of inoculum, % (v/v) <i>L. bulgaricus:Str. thermophilus</i> 3.8:1					<i>L. bulgaricus:Str. thermophilus</i> (v/v) inoculum					Serial inoculation with a mixed culture, 1 % (v/v)					
	2	1	0.5	0.25		T*	B†	0.6:1	1.5:1	3.8:1	49:1	T	B	1	2	3
Heat production, ‡ J mol ⁻¹	4.11	4.06	4.21	4.32		2.89	4.29	5.28	5.52	5.41	5.52	3.46	4.25	6.00	5.92	5.17
Maximum heat effect, μW ml ⁻¹	449	414	414	429	266	266	240	409	443	449	377	283	226	457	414	354
Time of maximum heat effect, h	3.4	3.4	4.0	4.4	2.8	2.8	6.1	2.8	3.5	3.8	4.7	2.5	6.6	4.1	3.5	3.0

* *Str. thermophilus* in single culture.

† *L. bulgaricus* in single culture.

‡ The heat production for the different ratios of strains in the inoculum were determined when the heat effect had descended to 150 μW/ml. All other values were integrated up to 9 h.

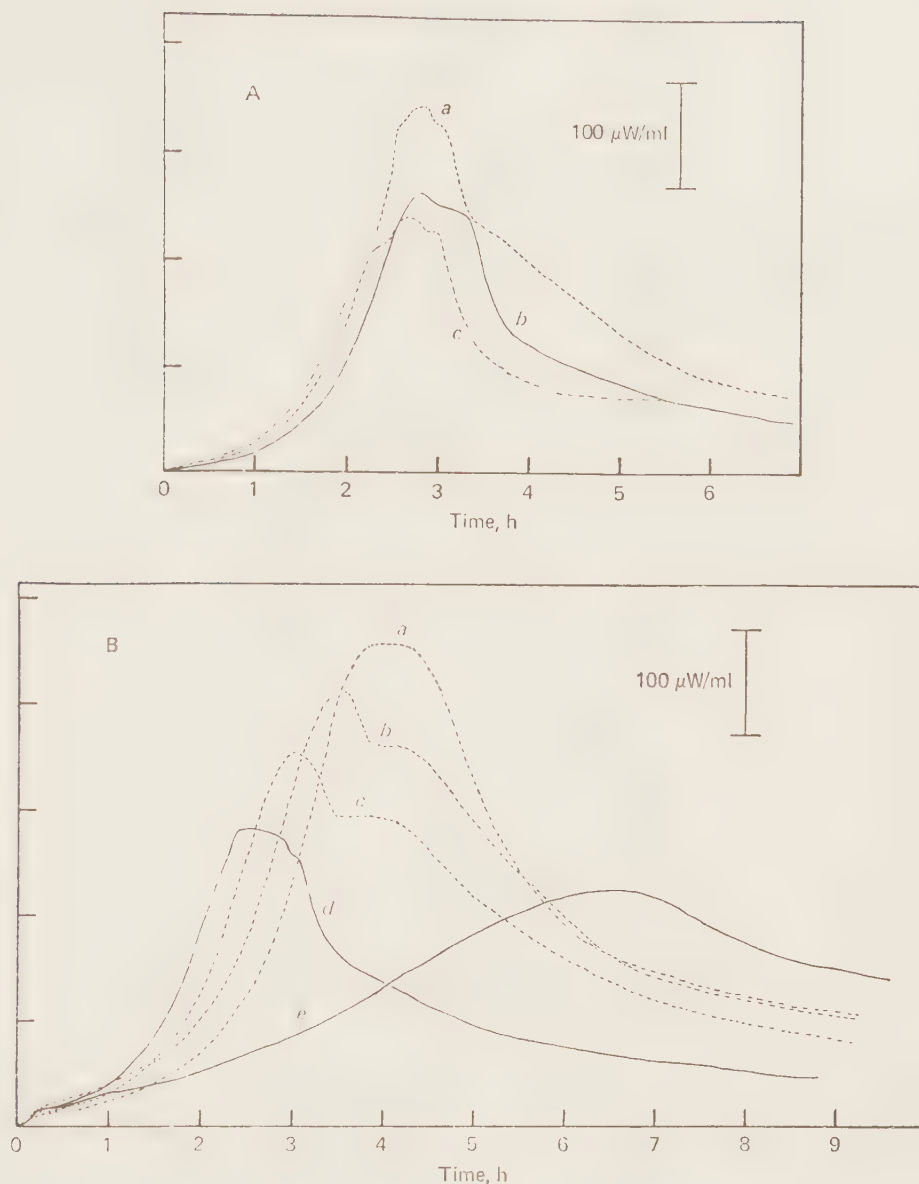


Fig. 2. (A) The effect of addition of 20 % (v/v), 8-h cell-free filtrates from *Lactobacillus bulgaricus* (a), and *Streptococcus thermophilus* (b), to 5 % (w/v) skim-milk medium inoculated with 1 % *Str. thermophilus*. Growth of *Str. thermophilus* without supplements (c). (B) Serial inoculation without pH control. Thermogram (a) was obtained with a 1 % inoculum of *L. bulgaricus* and *Str. thermophilus* in a ratio of 3.8:1. Nine hours inoculum from cultures giving (a) and (b) gave the thermograms (b) and (c). Growth of *Str. thermophilus* and *L. bulgaricus* gave (d) and (e).

and each successive mixed culture by (a), (b) and (c). The increase in heat effect produced by the 3 mixed fermentations could each be described by one first-order rate constant, while both the single cultures were described by 3 rates until the maximum heat effect had been reached.

The initial pH of the milk medium was 6.5; after growth of *Str. thermophilus* the pH was 4.5 and with *L. bulgaricus* pH 4.3. The effect of maintaining a constant pH

Table 2. *Heat production and maximum heat effect by Streptococcus thermophilus grown in milk medium supplemented with 20% (v/v) cell-free filtrates from 8-h cultures of Str. thermophilus and Lactobacillus bulgaricus. Effect of growth at pH 6.5 on the thermograms of the 2 strains compared with no pH control*

	Effect of cell-free filtrates on thermograms of <i>Str. thermophilus</i>			Effect of growth at pH 6.5 on thermograms			
	No addition	<i>Str. thermophilus</i> filtrate	<i>L. bulgaricus</i> filtrate	<i>Str. thermophilus</i> control	<i>Str. thermophilus</i> pH 6.5	<i>L. bulgaricus</i> control	<i>L. bulgaricus</i> pH 6.5
Heat production, J mol ⁻¹	2.54*	2.37*	3.42*	2.41†	2.45†	4.56†	5.52†
Maximum heat effect, μ W ml ⁻¹	263	240	346	269	274	220	286
Time of maximum heat effect, h	2.8	2.7	2.8	2.8	3.3	6.6	7.1

* Heat production after 7 h. † After 6 h. ‡ After 10 h.

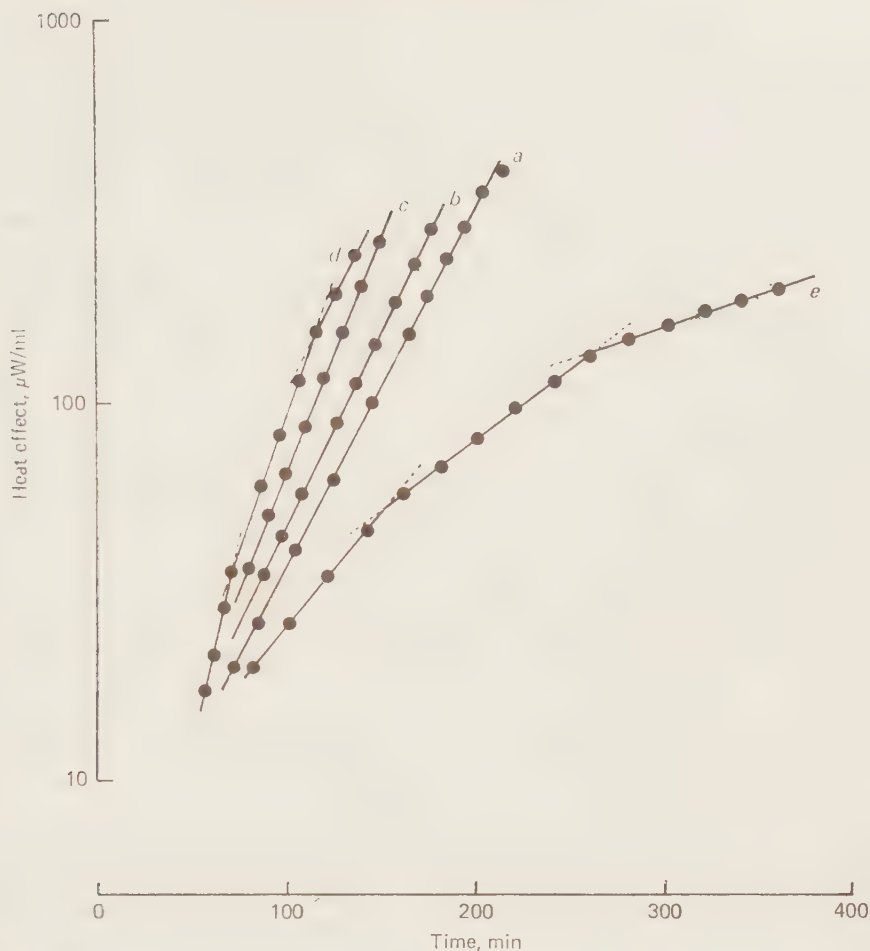


Fig. 3. The logarithmic increase in heat effect for the mixed cultures (a), (b), (c) and the single cultures (d) and (e) giving the thermograms shown in Fig. 2B.

of 6.5, compared with no control, on the fermentation by *Str. thermophilus* (a) and *L. bulgaricus* (b), can be seen from Fig. 4(A) and Table 2. No significant increase in either the heat production or maximum heat effect occurred when *Str. thermophilus* was grown at pH 6.5. *L. bulgaricus* showed a 21% increase in heat production and a 30% increase in the maximum heat effect.

Thermogram (a), Fig. 4(B), shows growth of a mixed culture at pH 6.5. A 1% (v/v) inoculum was used containing *L. bulgaricus* and *Str. thermophilus* in a ratio of 3.8:1. The maximum heat effect was 90% greater than the sum of the maximum heat effects found for the single cultures grown at pH 6.5. Thermograms (b) and (c) were obtained by using inocula from the fermentations giving the thermograms (a) and (b) respectively. The maximum heat effect was significantly reduced on repeated subculture. All the thermograms were more profiled than those obtained without pH control.

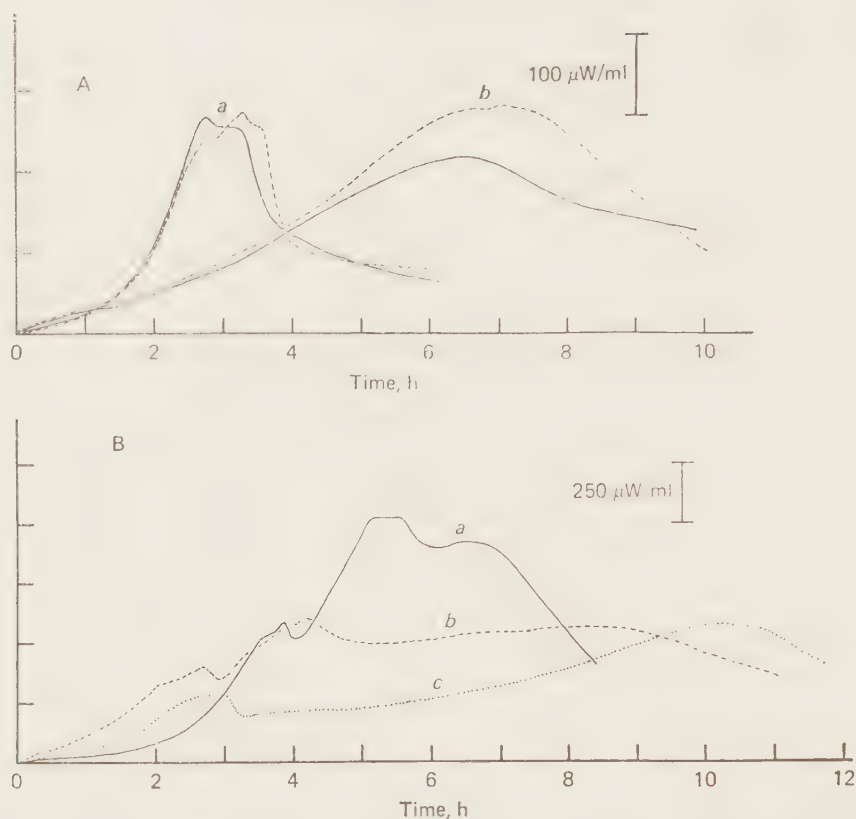


Fig. 4. (A) Thermograms of *Streptococcus thermophilus* (a) and *Lactobacillus bulgaricus* (b), ----, grown at pH 6.5; —, without pH control. (B) Growth of mixed cultures at pH 6.5. Thermogram (a) was obtained with a 1% inoculum of *L. bulgaricus* and *Str. thermophilus* in a ratio of 3.8:1. Thermograms (b) and (c) were obtained with inocula from the fermentations giving (a) and (b) respectively.

DISCUSSION

A thermogram of microbial growth is a continuous record of the rate of heat production. The area enclosed by a thermogram is proportional to the heat produced and has 3 main components, catabolism of the substrates supplying energy, heats of dilution and ionization of the reaction products and the enthalpy changes associated with growth. Catabolism of milk sugars, essentially lactose, will supply the energy for growth of lactic acid bacteria in milk medium, and will give rise to the largest amount of heat. The heat production from catabolism will depend upon the stoichiometry of the carbohydrate degradation and be directly related to the amount of products formed, mainly lactic acid.

The magnitude of the heat effect at any time will be related to the cell density, the rate of catabolism per unit mass and the enthalpy change of the catabolic degradation. The heat effect will decrease when growth stops, substrate concentration or a growth factor limits growth or the rate of catabolism decreases due to inhibition by metabolites, including a change in pH. The maximum heat effect reached during growth will be a measure of the cell yield. The rate of increase in heat effect for balanced growth will have the same doubling time as the growth rate, degradation of substrate and the formation of products (Monk, 1978). In a mixed

culture, the heat effect and the quantity of heat at any time will be the sum of the contributions by each component strain.

The thermograms of the individual cultures show *Str. thermophilus* to grow much more rapidly and to complete its metabolism earlier than *L. bulgaricus*. It is seen from Fig. 4(A) that growth at pH 6.5 compared with no control has almost no effect on the heat effect or heat production of *Str. thermophilus*, while *L. bulgaricus* grows much more successfully when the acid produced is neutralized at pH 6.5. With the strains used, the growth of *L. bulgaricus* could be inhibited by acid production from the faster growing *Str. thermophilus*. Moon & Reinbold (1976) reported that *L. bulgaricus* in mixed culture was inhibited both during exponential growth and in the stationary phase, although the effect was attributed to competition for essential nutrients rather than a reduction in pH. The thermograms of mixed cultures showed an interaction between the 2 strains much more complex than the sum of the heat effects for the individual thermogram, e.g. Fig. 2(B). At the optimum inoculum conditions to obtain a maximum heat effect in mixed culture, the heat production was about 80 % of the additive values for the individual cultures, while the maximum heat effect was 90 % (Table 1). Increases in growth and acid production have been reported for these bacteria and attributed to compounds produced by *L. bulgaricus* which stimulate the growth of *Str. thermophilus* (Shankar & Davies, 1977). The extent of the stimulation can be seen from Fig. 2(A). As growth of *Str. thermophilus* was not limited by either carbohydrate or pH, the stimulation is attributed to growth factors provided by the medium in which *L. bulgaricus* had grown.

At a constant ratio of *L. bulgaricus* to *Str. thermophilus* of 3.8:1, the amount of inoculum only influenced the lag time of the fermentation, Fig. 1(A), without affecting either the maximum heat effect or the amount of heat produced. The stimulation of the growth of *Str. thermophilus* remained constant, the growth of the mixed culture behaved as a single culture grown with different amounts of inoculum. The composition of the inoculum had a much greater effect on the progress of the fermentation although the heat production did not change significantly, Fig. 1(B), Table 1. The maximum heat effect was obtained with an inoculum containing a ratio of *L. bulgaricus* to *Str. thermophilus* of 3.8:1. Moon & Reinbold (1976) reported a ratio of 2:1 for the maximum stimulation of growth of *Str. thermophilus* in mixed culture. With increasing numbers of *Str. thermophilus* in the mixed inoculum the maximum heat effect was reached much earlier with only a small decrease compared with the maximum value. The small amount of growth of *L. bulgaricus* which must have occurred before the maximum heat effect was reached was still sufficient to stimulate the growth of *Str. thermophilus*.

On repeated subculture without pH control (Fig. 2B), the decrease in maximum heat effect and heat production together with a decrease in lag time indicates the increasing dominance by *Str. thermophilus* in the mixture and the poor reproducibility of the fermentation. Mixtures have been reported which are particularly stable over a wide range of ratios of cell numbers between strains of these 2 organisms (Accolas *et al.* 1977). When the mixture was grown with pH control, the maximum heat effect was stimulated to almost double the sum of that for the individual cultures and the interaction between the 2 strains became complex (Fig. 4B). When examined by repeated subculture the maximum heat effect showed a large decrease and the thermograms were irregular when compared with repeated subculture without pH control.

A single logarithmic expression of the increase in heat effect associated with the growth of *Str. lactis* can be used to describe balanced growth (Monk, 1978). In Fig. 3 the mixed cultures show a single logarithmic increase in heat effect while the single cultures are each described by 3 rates. In mixed cultures, this condition requires that both strains are growing exponentially, an indication that mixed growth improves the nutritional environment for both bacteria. At each successive sub-culture *a*, *b*, *c*, the increase in heat effect is seen to increase, a further indication of the increasing dominance of *Str. thermophilus* in the mixture.

Together with analytical information a thermogram can be used to define metabolism in thermochemical terms; however, it has the added advantage of presenting a pictorial record of the overall metabolism. The observations on the interaction of *Str. thermophilus* and *L. bulgaricus* demonstrate the range of parameters amenable to this technique. With currently available instrumentation, only one calorimetric measurement can be made at a time, a limitation with microbiological work where controls in parallel are often essential. The application of calorimetry to applied microbiology will depend upon the availability of a relatively inexpensive, multi-channel instrument.

Further work in development of the technique for selection of cultures for growth compatibility and their resistance to phage infection is in progress.

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IX

Catabolism of Glucose by Streptococcus faecalis and Streptococcus agalactiae in the presence of 2,4-Dinitrophenol, Anaerobically and Aerobically as Affected by pH.

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SUMMARY

Washed suspensions of Streptococcus faecalis and Streptococcus agalactiae show increased glucose catabolism depending upon the concentration of 2,4-dinitrophenol (DNP) and the age of the cells when harvested. The maximum stimulation by DNP is similar to the initial transitory rate observed on the addition of glucose to untreated suspensions. Anaerobically without DNP, both strains maintain a constant glycolytic rate essentially independent of pH, with DNP present the glycolytic rate responds to buffer pH. The presence of oxygen does not change the rate of glucose catabolism by untreated suspensions, but the rate of oxidation of metabolically formed pyruvate depends upon buffer pH for S. faecalis, but not S. agalactiae. The pH within the cell appears to be implicated in regulating the rate of glucose catabolism. A calorimetric method has been used to measure the aerobic and anaerobic catabolism of glucose.

INTRODUCTION

The rate at which glucose is degraded by washed suspensions of many lactic acid bacteria is a well defined property (2,9). However, transitory elevated rates of glycolysis have been reported for K^+ depleted cells of Streptococcus faecalis associated with K^+ accumulation and Na^+ extrusion (15). The heat production by S. faecalis on the addition of glucose has also shown an initial elevated rate, which then changed to a lower rate. Anaerobic conditions were maintained and a homolactic fermentation was observed for both rates of glucose catabolism and the response was not affected by the type of buffer cation (4).

The addition of proton conductors to suspensions of S. faecalis (6,12) and Streptococcus lactis (8) catabolizing glucose causes an increased rate of glucose degradation, an affect which occurs anaerobically and aerobically without affecting the substrate level generation of adenosine-5-triphosphate (ATP), or its utilization (7). It has been suggested that the increased rate is regulated by the ATP level in the cell. Uncouplers cause an efflux of H^+ which can stimulate ATP-ase activity and in response to the decrease in ATP, glycolysis is stimulated to maintain a steady state (8). However, a large variation in the ATP pool has been observed in S. faecalis without affecting the rate of glycolysis even under conditions where some ATP is utilized in synthetic reactions (3).

The addition of glucose to a cell suspension or an uncoupler to cells degrading glucose both influence the pH within the cell. Uncouplers act on the cell membrane causing the collapse of the pH gradient between the buffer and the cell, and the addition of glucose increases the cell pH relative to the buffer (6). It is therefore possible that a change in pH within the cell could be involved in the regulation of glycolysis.

In this investigation some properties of the uncoupler 2,4-Dinitrophenol (DNP), in stimulating glucose catabolism by washed suspensions of S. faecalis and Streptococcus agalactiae have been examined under anaerobic and aerobic conditions. The affect of buffer pH on the rate of glucose catabolism in the presence of DNP has been compared with untreated suspensions. These observations have been interpreted in relation to the expected pH within the cell and with the initial elevated rate of glucose catabolism measured on the addition of glucose to washed cell suspensions.

MATERIALS AND METHODS

Bacteria. Streptococcus faecalis (ATCC 4083) and a human faecal isolate, a β -haemolytic Streptococcus belonging to Lancefield group B (Streptococcus agalactiae) were used.

Growth conditions and preparation of washed suspensions. Cultures were grown at 37°C on a complex medium containing one percent each of glucose, trisodium citrate, peptone and yeast extract. The incubation time was usually 16 h. The cells from 1 litre of medium were harvested by centrifugation and washed twice with 0.2 M (KH_2PO_4 - K_2HPO_4) buffer at pH 6.2. The cell pellet was resuspended in 50 ml of similar buffer. Cell suspensions for experiments were prepared by dilution with buffers of different pH containing glucose and uncoupler to cell densities between 0.5 and 1 mg/ml. The cell suspensions were centrifuged immediately after the addition of glucose to a concentration of 10 mM and the pH of the supernatant was measured with a Radiometer digital pH meter PHM 52 at 37°C.

Cell densities were determined by measuring the optical density of suspensions at 650 nm in a Shimadzu spectrophotometer QV 50 and comparing the values with dry weight calibration curves prepared from stationary

phase cells of each culture. The cell density of suspensions prepared from S. faecalis during growth on complex medium to determine the affect of DNP on glucose catabolism were obtained after washing with water and drying to a constant weight at 100°C.

Measurement of anaerobic and aerobic glucose catabolism. Glucose catabolism was measured at 37°C in a flow calorimeter (11), having a gold measuring vessel of 0.65 ml. Cell suspensions containing 10 mM glucose were mixed and aerated at room temperature with water saturated compressed air, and pumped through the calorimeter at 40 ml/h. After obtaining a steady state thermal output, about 15 min, the flow was stopped and another lower steady state representing anaerobic conditions was established after several minutes.

The calorimetric method was unsuitable for measuring the initial catabolic rate because of the delay in transporting the suspension to the calorimetric vessel. Two similar cell suspensions of S. agalactiae or S. faecalis in 0.2 M potassium phosphate buffer, pH 6.2 were continuously aerated with water saturated air and incubated at 37°C. One suspension contained 250 µM DNP and to initiate catabolism, glucose was added to both suspensions to a concentration of about 6 mM. Samples were removed initially at 1 min and then at 3 min intervals for glucose analysis. Measurements were also made with two similar cell suspensions under anaerobic conditions, maintained by gassing with water saturated nitrogen. One milliliter samples for glucose analysis were dispensed into 2 ml of 8 % (vol/vol) perchloric acid and centrifuged, and glucose in the supernatant was determined.

The formation of lactic acid from glucose catabolism by S. agalactiae in the presence of DNP was measured under both anaerobic and aerobic conditions. Buffer containing 250 µM DNP and about 6 mM glucose was prepared in 5 replicates of 25 ml. The suspensions were gassed with water saturated nitrogen for 3 min before initiating catabolism by adding 1 ml

of a washed cell suspension to obtain a cell density of 0.8 mg per ml. Glucose concentration was determined before the addition of the cell suspension and after incubation at 37°C for 2 hours in sealed bottles. Lactic acid concentration was determined after fermentation.

Glucose was determined by the GOD-Perid method (Boehringer, Mannheim, Germany) and lactic acid with lactic acid dehydrogenase (Sigma Chemical Company, St. Louis, U.S.A.).

RESULTS

The calorimetric record of aerobic and anaerobic heat production.

Fig. 1 demonstrates the heat production recorded for the catabolism of glucose by S. faecalis. Similar results were obtained with S. agalactiae.

An initial baseline was established with buffer and on presentation of a cell suspension a small increase is observed (a), due to endogenous metabolism. On addition of substrate to the suspension the calorimeter, after a small lag time, responds to the increased heat production showing a steady state (b). When 250 μ M DNP was added the heat output increased to a new steady state (c). On stopping the flow, indicated by an arrow, a small increase was observed (d), corrected for in calculations by using electrical calibration constants obtained at flow and stopped flow. After several minutes the record decreased slowly (e) and then rapidly to a new steady state (f), which was subsequently found to show the steady state homolactic catabolism of glucose under anaerobic conditions. At subsequent measurements the response with and without DNP was obtained with paired suspensions.

Calorimetric measurement of glucose degradation. Washed suspensions of S. faecalis ATCC 4083 give a homolactic fermentation of glucose under anaerobic conditions and calorimetry can be used to quantitate this reaction (2). The steady state heat production established for S. agalactiae

after stopping the flow of suspensions containing glucose was compared with the expected enthalpy change for a homolactic fermentation. In Fig. 2 linear relationships are shown between the anaerobic and aerobic heat production and cell density for *S. agalactiae* catabolizing glucose.

The slope of the regression for the anaerobic relationship gives 150 μW per mg dry weight of cells and the mean value for glycolysis from 5 measurements was 0.082 μM glucose per min. per mg. These two results lead to an enthalpy change for glycolysis of -107 kJ per mole of glucose. The calculated value for the homolactic fermentation in 0.2 M potassium phosphate buffer, pH 6.2 is -117 kJ per mole of glucose (12). The measured and calculated values show good agreement, allowing subsequent results given as thermal output to be considered directly proportional to the anaerobic rate of glucose degradation. The aerobic results show modification due to oxidation of pyruvate (1). The anaerobic heat production by both strains could therefore be used to measure the rate of glucose catabolism to lactic acid.

The effect of DNP concentration on glucose catabolism by *S. faecalis* and *S. agalactiae*. Suspensions of *S. faecalis* and *S. agalactiae* in 0.2 M potassium phosphate buffer, pH 6.2 were treated with different concentrations of DNP. Glucose was added to 10 mM and the anaerobic heat production was recorded at each level of DNP and the results plotted in Fig. 3. The significant difference between the two bacteria was the dependence of glycolytic stimulation on DNP concentration. The effect reached saturation below 100 μM DNP for *S. faecalis* while for *S. agalactiae* the maximum stimulation was approached at about 300 μM DNP. *S. agalactiae* was also examined at one half the cell density (0.6 mg/ml), and similar results were obtained for glycolytic stimulation. The action of DNP was not dependent upon cell density.

Reversibility of DNP action on glycolytic stimulation. A cell suspension of *S. faecalis* in potassium phosphate buffer containing 250 μM DNP

and 10 mM glucose showed stimulated heat production when compared with untreated cells. A similar suspension treated for 30 min with 250 μ M DNP and 10 mM glucose, which was then centrifuged and the cells resuspended in fresh buffer without DNP, but containing glucose, gave similar results as the control. Results summarized in Table 1 show that after treatment with DNP the cells regain their catabolic rate, the affect of DNP being reversible.

Action of DNP on catabolism of glucose by *S. faecalis* harvested at different stages of growth. A culture grown in complex medium at 37°C was used for preparing washed cell suspensions during exponential growth and the stationary phase. Suspensions ranging from 0.5 to 1.0 mg per ml in 0.2 M potassium phosphate buffer at pH 6.2 were measured for stimulation of anaerobic glycolysis by 250 μ M DNP. The results are shown in Table 2. The ability of DNP to stimulate glycolysis at the beginning of exponential growth was small, increasing during growth and continued to increase in the stationary phase.

Transient rates of glucose catabolism by washed cell suspensions. In the absence of DNP both the aerobic and anaerobic suspensions of *S. agalactiae* degrade glucose in two well defined rates. Both sets of data are shown as one line, Fig. 4A, but glycolytic rates were calculated separately, Table 3. The initial elevated rate continues for 8 to 10 min after glucose addition when the rate then decreases abruptly. In the presence of DNP (Fig. 4b) the rate of glucose degradation continues at the initial rate. Measurements with *S. faecalis* did not show a change in rate of glucose degradation in the absence of DNP, however the rate was elevated in the presence of DNP. The rates of glucose degradation for the different experimental conditions are shown in Table 3 with the coefficient of determination and the number of experimental values used to fit the regression. The initial rates of glucose degradation both anaerobically and aerobically for *S. agalactiae* are the same as those measured for the

single rate in the presence of DNP. At the concentration giving its maximum effect DNP raises and maintains the level of glucose degradation to that found initially on the addition of glucose. The final rates both anaerobically and aerobically in the absence of DNP are also similar.

Lactic acid formation from glucose catabolism anaerobically and aerobically with and without DNP present. The formation of lactic acid from glucose catabolism by S. agalactiae with and without 250 μM DNP present in phosphate buffer at pH 6.2 gave for the mean value of 5 measurements,

Without DNP; 1 glucose $\longrightarrow$ 1.93 lactate

With DNP; 1 glucose $\longrightarrow$ 1.89 lactate

There was no significant difference between the two reactions at the 90 per cent confidence interval and DNP had no effect on the amount of lactic acid formed in the anaerobic fermentation. The reaction was essentially homolactic in both cases. The measurements were repeated under aeration using water saturated air at 37°C. The fermentations gave,

Without DNP, 1 glucose $\longrightarrow$ 1.47 lactate

With DNP, 1 glucose $\longrightarrow$ 1.62 lactate

By substituting the rates of aerobic degradation of glucose by S. agalactiae from Table 3 into the quantitative relationship for aerobic glucose degradation gives the rate equation shown below, per minute.

Without DNP; 0.085 μM glucose $\longrightarrow$ 0.125 μM lactate

With DNP; 0.106 μM glucose $\longrightarrow$ 0.172 μM lactate

The results show that the measured rate of glucose degradation stimulated by DNP is shunted to lactate.

Effect of pH and oxygen on glucose catabolism with DNP present. In Fig. 5 is shown the effect of 250 μ M DNP on the anaerobic and aerobic catabolism of glucose by S. faecalis in 0.2 M potassium phosphate buffer at different pH values. The results are the accumulated data obtained with at least three different cell preparations. The anaerobic catabolism of glucose was constant in the absence of DNP over the pH range studied, 5.3 to 6.8. With DNP present the steady state heat production increased to a maximum at pH 6.2 and then slowly decreased with increasing pH. The aerobic heat production with DNP present gave a pH dependent response similar to that obtained in its absence, but stimulated due to increased glucose degradation.

In Fig. 6 the results of corresponding treatments with S. agalactiae are summarized. The heat production both anaerobically and aerobically without DNP remains constant over the measured pH range 5.6 to 6.6. With 250 μ M DNP present both the anaerobic and aerobic reactions showed a maximum rate of heat production at pH 6.0.

DISCUSSION

Washed suspensions of S. faecalis and S. agalactiae gave a homolactic fermentation of glucose under anaerobic conditions. Similar to observations on S. faecalis (2), this was shown by thermochemical calculations on glucose catabolism by S. agalactiae and confirmed by analytical measurements. Aerobic fermentations of glucose by both cultures give a mixture of products due to the oxidation of some pyruvate (1). These reactions are more exothermic than the formation of lactate and calorimetry is a sensitive indicator of changes from aerobic to anaerobic catabolism. This is shown in Figs. 1 and 2.

The stimulation of heat production by the action of DNP on both cultures fermenting glucose, increased with DNP concentration, but the concentration required to achieve the maximum response was different, Fig. 3. The increase in heat production at each level of DNP was the same, both anaerobically and aerobically. A homolactic fermentation was maintained anaerobically in the presence of DNP, hence the heat production showed that the glucose degraded appeared as lactate independent of the rate of glucose catabolism or the presence of oxygen at pH 6.2.

This was confirmed for S. agalactiae by analytical measurements.

Regulation of glucose catabolism occurs in S. agalactiae several minutes after glucose addition, Fig. 4A and the initial higher catabolic rate is maintained by DNP when added at a concentration showing maximum stimulation, Fig. 4B and Table 3. This occurs both anaerobically and aerobically. S. faecalis (ATCC 4083) also shows a similar regulation, but the change between the two rates occurs within a minute of glucose

addition, (W.W. Forrest, personal communication). The sampling methods used in this work were not rapid enough to observe this change. The similar maximum rates of glucose catabolism observed initially on the addition of glucose and maintained by DNP action indicates a common factor in the regulation of the catabolic rate.

The formation of ATP from different rates of glucose catabolism is unlikely to cause the observed regulation and no change in rate of glucose catabolism was found aerobically where higher yields of ATP are expected from pyruvate oxidation (1).

One common factor between the two rates of glucose catabolism and the action of DNP is a change in pH within the cell. Resting cells assume the same pH as the buffer, but when degrading glucose a constant internal pH more alkaline than the buffer is established, while DNP acting as a proton conductor can equilibrate the cell pH with that of the buffer.

Heat production from glucose catabolism by S. faecalis in response to buffer pH is shown in Fig. 5. Over the pH range 5.3 to 6.8 the corresponding pH range within the cell is expected to be 6.7 to 7.6 (6). No difference in anaerobic heat production was observed over this pH and the rate of glucose catabolism was therefore constant and as previously discussed, homolactic. However with DNP present the observed heat production varied with buffer pH. This could occur if the uncoupling activity of DNP was pH dependent, the products of anaerobic catabolism varied with buffer pH or the action of DNP as a proton conductor changed the rate of glucose catabolism in response to buffer pH.

The activity of uncoupling agents are pH dependent and usually show a maximum activity at a pH more alkaline than their pK in aqueous solution (14), however with DNP ($pK=4.0$), decreasing the pH has shown its uncoupling activity to be optimized at lower concentrations (5). The concentration of DNP used was in excess of that required for the maximum stimulation of glucose catabolism by S. faecalis at pH 6.2. Fig. 3, therefore a lower pH should not reduce its activity as shown in Fig. 5.

S. agalactiae shows a similar response with buffer pH, Fig. 6, as S. faecalis in the anaerobic catabolism of glucose in the presence of DNP. However no significant changes in the products formed by S. agalactiae growing on glucose have been found due to DNP activity, where the pH of the fermentation will change during growth (10). One observation for S. faecalis, Fig. 5, is lower than the heat production for the anaerobic homolactic fermentation, which can only occur with a decreased rate of glucose catabolism. If DNP equilibrates the cell and buffer pH the results obtained with DNP present will be at pH values lower than those observed in its absence. At low pH the rate of glucose catabolism is expected to decrease. These results indicate that buffer pH is not affecting the uncoupling activity of DNP or changing the products of fermentation, but having a direct influence on the rate of glucose catabolism.

In the presence of oxygen the heat production from glucose catabolism by S. faecalis, Fig. 5 changes with buffer pH, while S. agalactiae, Fig. 6 is not affected. The rate of glucose catabolism does not change in response to oxygen, Table 3 or buffer pH without DNP present over the range examined for either S. faecalis or S. agalactiae. The observed change in heat production by S. faecalis in the presence of oxygen indicates a variation in the balance of products formed from pyruvate in response to buffer pH. A reduced rate of heat production with decreasing pH is expected from an increase in the proportion of lactate formed from pyruvate. Lactate dehydrogenase from group N streptococci is activated by fructose-1,6-diphosphate in vitro and is stimulated by decreasing pH (13) an affect which might explain the in vivo observations with S. faecalis. The constant aerobic heat production by S. agalactiae suggests that both the rate and proportion of the products formed from pyruvate remains the same independent of pH changes in the cell in response to buffer pH.

With both DNP and oxygen present the heat production by *S. agalactiae* Fig. 6, is stimulated by the presence of oxygen and the action of DNP causes the rate of glucose catabolism to be directly influenced by buffer pH. With *S. faecalis*, Fig. 5 the heat production is determined both by the rate of glucose catabolism in response to buffer pH and the affect of oxygen on product formation with changing buffer pH.

The stimulation of glucose catabolism by *S. faecalis* with DNP depends upon the stage of growth, Table 2, while its action on the cell is reversible, Table 1. DNP acts on the cell membrane and its ability as a proton conductor depends upon the concentration of the anionic form in the lipid phase (5). The increase in its activity depending on the stage of growth at which the cells are harvested may be related to the changing lipid composition of the cell wall.

In conclusion the increased glucose catabolism by bacteria in the presence of DNP appears to be determined by its action in changing the internal pH of the cell while the extent of the stimulation is determined by the strain and the experimental conditions used to make the observation.

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TABLE 1.

Reversible stimulation of glucose catabolism by S. faecalis with 250 μ M DNP in 0.2 M phosphate buffer, pH 6.2.

Treatment	Anaerobic heat	Aerobic heat
	effect μ W	effect μ W
glucose without DNP	126	210
glucose with DNP	230	308
glucose with DNP for 30 min, then glucose without DNP	126	205

TABLE 2.

Stimulation by 250 μ M DNP of glycolysis by S. faecalis harvested at different stages of growth.

Hours after inoculation	Stage of growth	Percentage stimulation of anaerobic heat effect
2,3	1,4 generations ^a	5
4,5	2,5 generations ^a	16
6	Early stationary	41
8	Mid stationary	44
16	Late stationary	56

^a Generations were calculated from beginning of exponential growth.

TABLE 3.

Rates of glucose degradation by *S. agalactiae* and *S. faecalis* anaerobically and aerobically in the presence and absence of 250 μ M DNP in 0.2 M potassium phosphate buffer pH 6.2 at 37°C.

Treatment	<i>S. agalactiae</i>		<i>S. faecalis</i>	
	Glucose	Coefficient of	Glucose	Coefficient of
	degraded	determination ^a	degraded	determination ^a
	μ M/min/mg		μ M/min/mg	
Anaerobic, no DNP				
Initial rate	.118	.988 (10)	.096	.997 (20)
Final rate	.079	.996 (8)		
Anaerobic, with DNP	.114	.994 (20)	.145	.994 (20)
Aerobic, no DNP				
Initial rate	.117	.980 (8)	.086	.992 (19)
Final rate	.085	.988 (11)		
Aerobic, with DNP	.106	.995 (19)	.134	.995 (18)

^aNumber of values used to calculate rates are shown in parenthesis.

FIGURE LEGENDS

Fig. 1. Thermograms of S. faecalis catabolizing glucose in 0.2 M potassium phosphate buffer. Endogenous metabolism a, aerobic catabolism b, response to 250 μ M DNP c, oxygen limitation e, and anaerobic catabolism with DNP present f. The arrow indicates the time at which the flow was stopped followed by d, showing an increased heat production corrected for by the calibration constant in calculations.

Fig. 2. Correlation between heat production and different cell suspensions of S. agalactiae in 0.2 M potassium phosphate buffer, pH 6.2. Symbols: $\circ$, aerobic; $\bullet$, anaerobic.

Fig. 3. The effect of DNP concentration on glucose catabolism in 0.2 M potassium phosphate buffer, pH 6.2. Symbols: $\blacksquare$, S. faecalis anaerobic and $\square$, aerobic; $\bullet$, S. agalactiae anaerobic and $\circ$, aerobic. Cell density 1 mg/ml.

Fig. 4. The rate of glucose catabolism by S. agalactiae in 0.2 M potassium phosphate buffer, pH 6.2.
Symbols: A without DNP; $\bullet$, anaerobic and $\circ$, aerobic.
B with 250 μ M DNP; $\bullet$, anaerobic and $\circ$, aerobic.

Fig. 5. The effect of pH on the catabolism of glucose by S. faecalis in 0.2 M potassium phosphate buffer.
Symbols: $\blacksquare$, anaerobic without DNP and $\bullet$, with 250 μ M DNP; $\square$, aerobic without DNP and $\circ$, with 250 μ M DNP.

Fig. 6. The effect of pH on the catabolism of glucose by S. agalactiae in 0.2 M potassium phosphate buffer. Symbols: ■ , anaerobic without DNP and ●, with 250 μ M DNP; □ , aerobic without DNP and ○, with 250 μ M DNP.

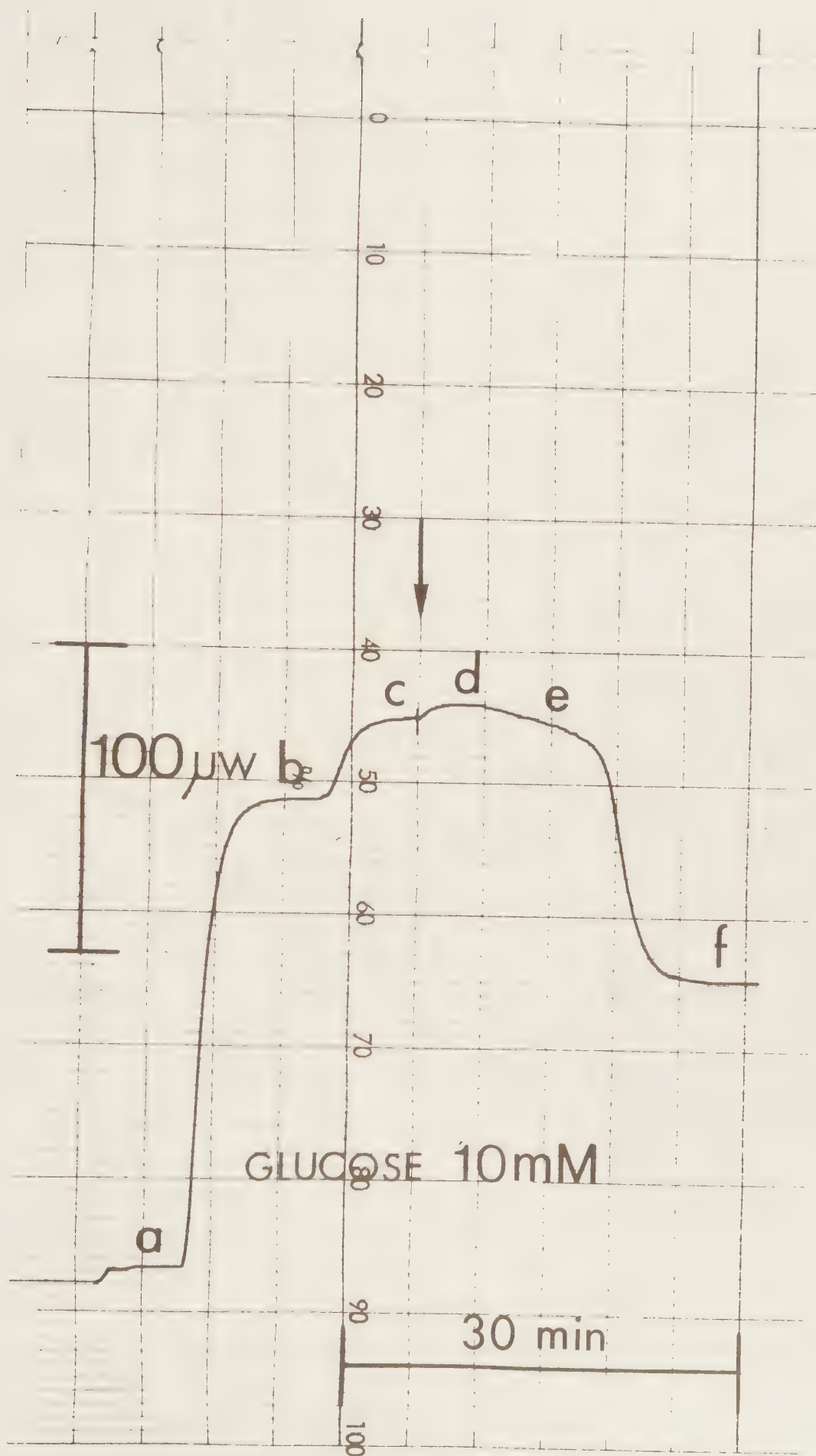


Fig 1

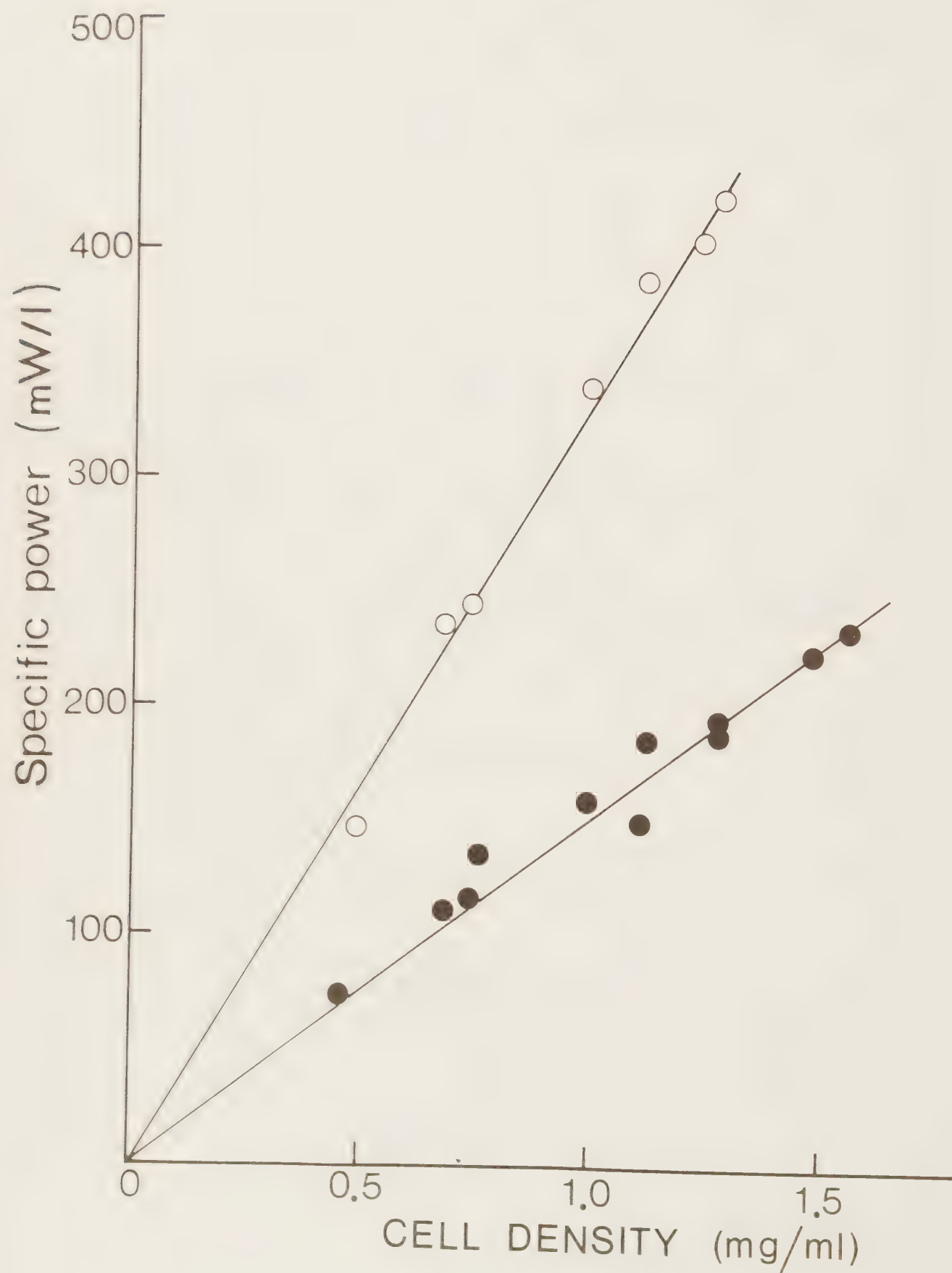


Fig 2

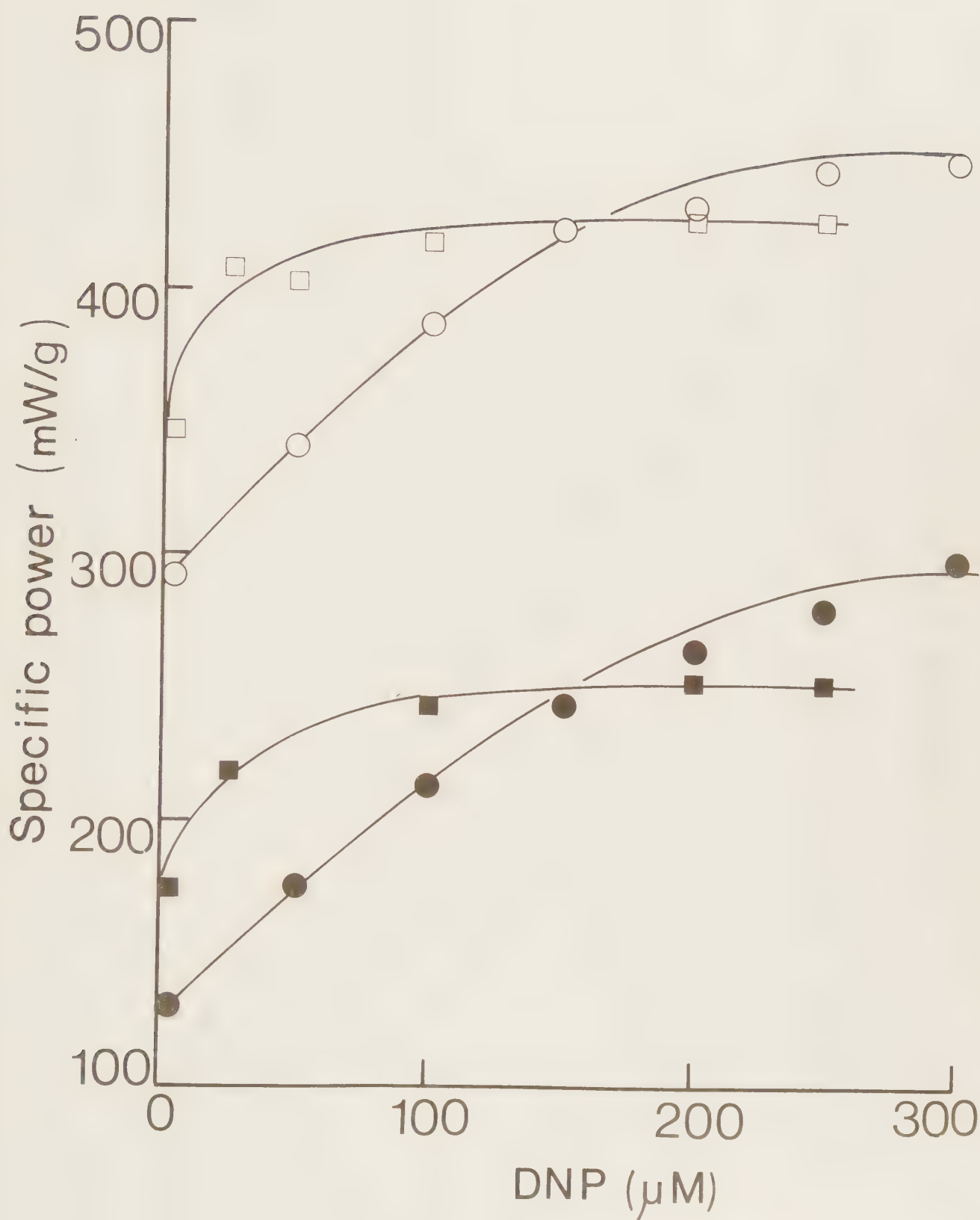


Fig 3

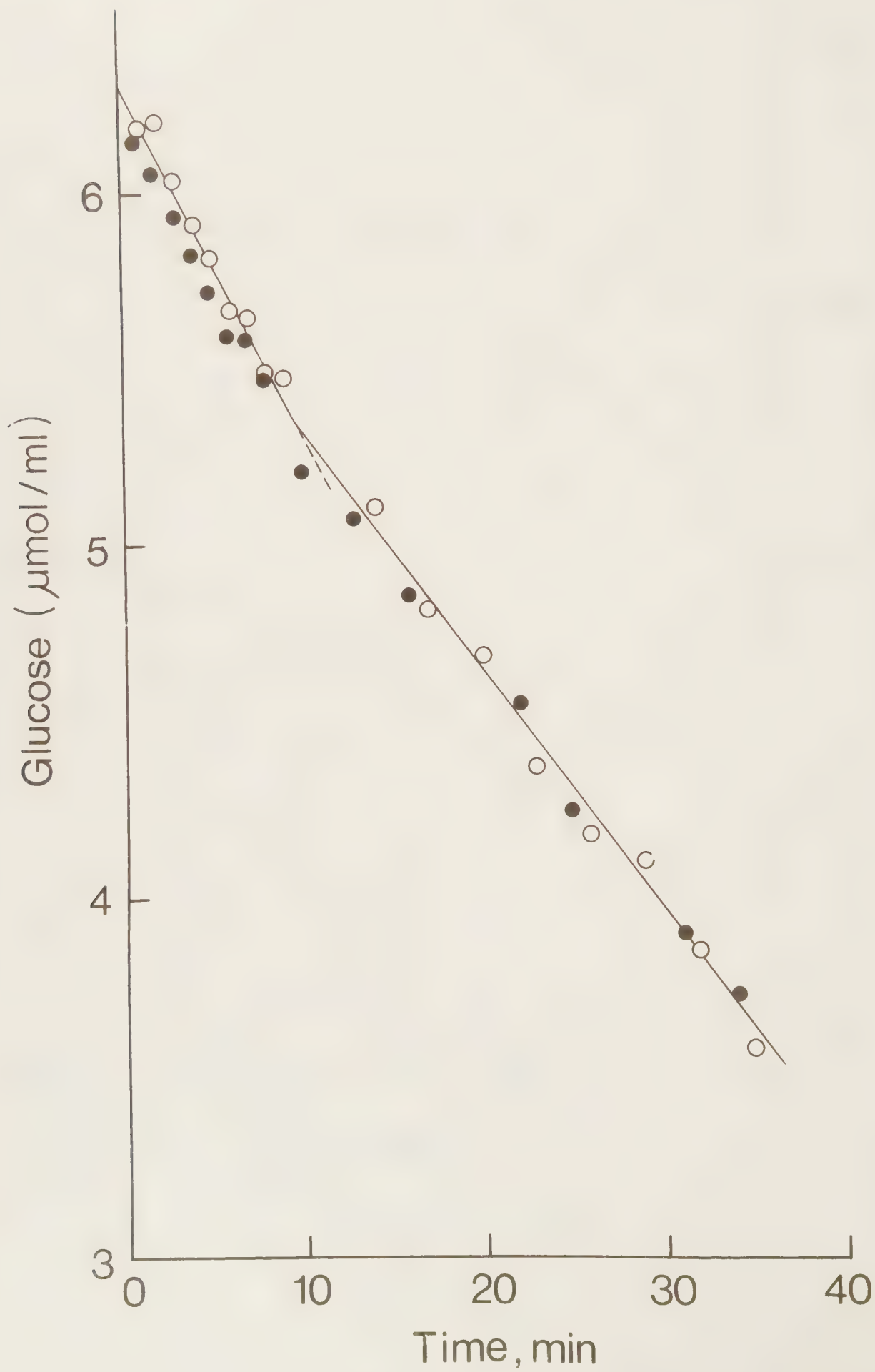


Fig 4A

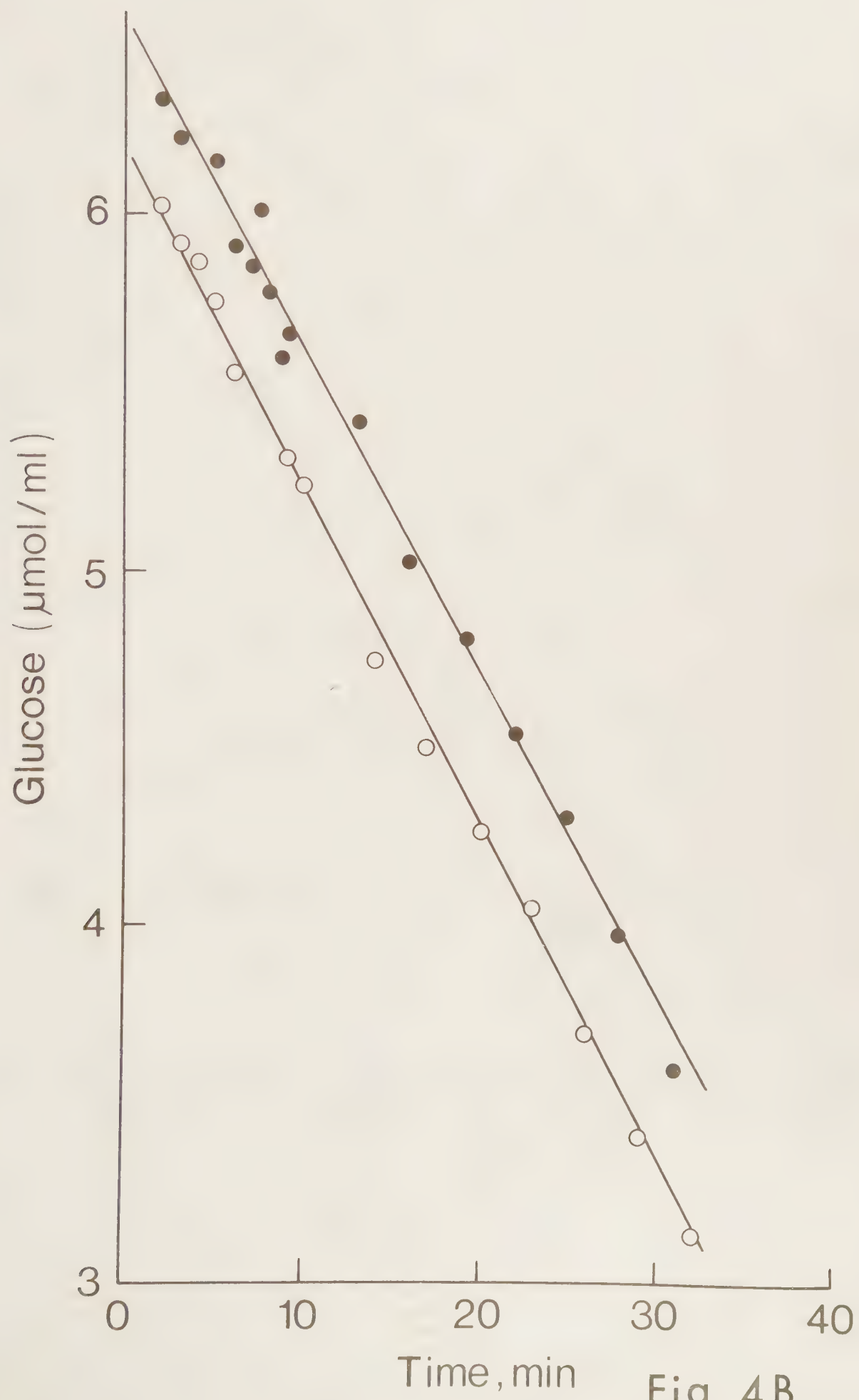


Fig 4B

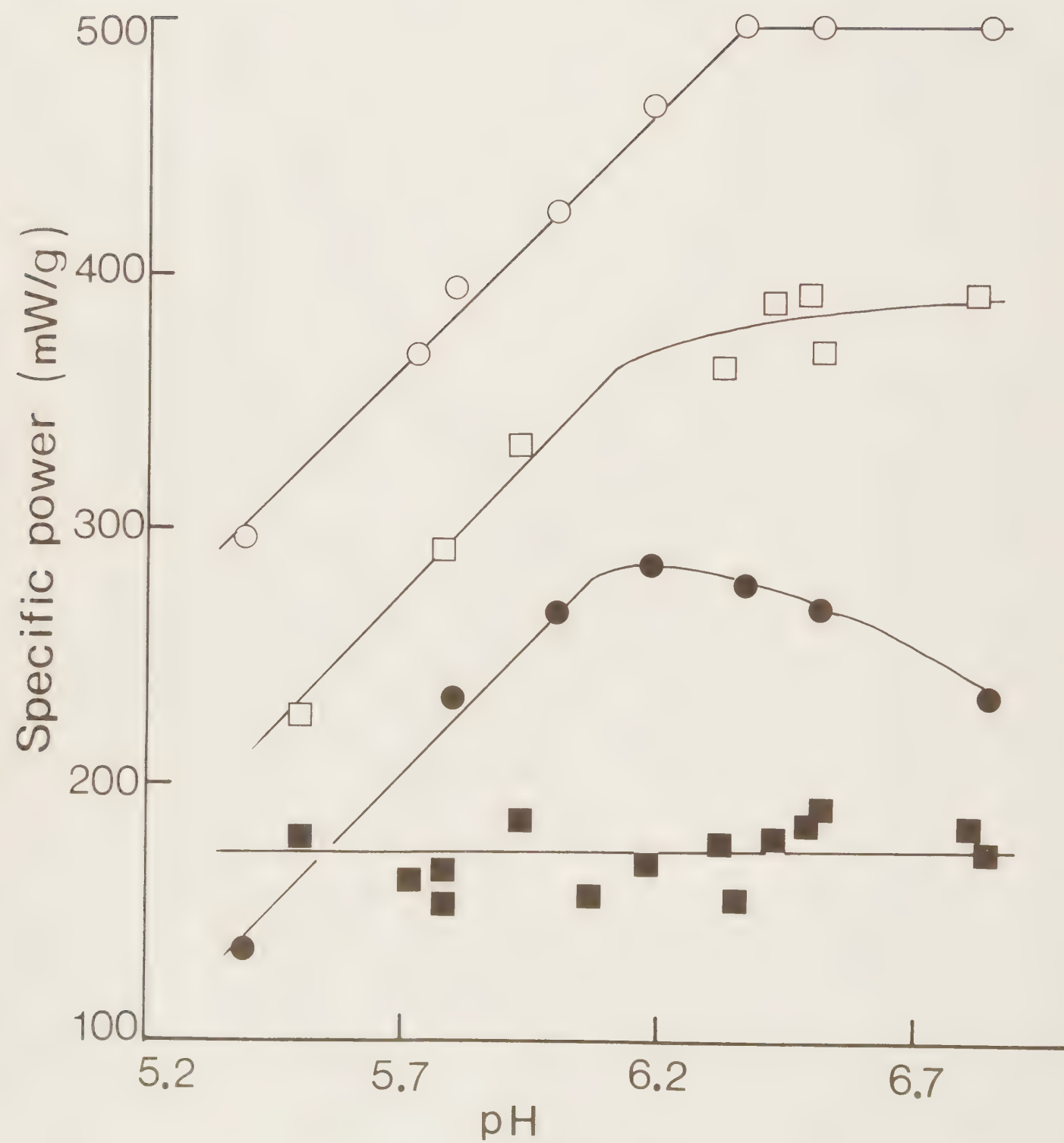


Fig 5

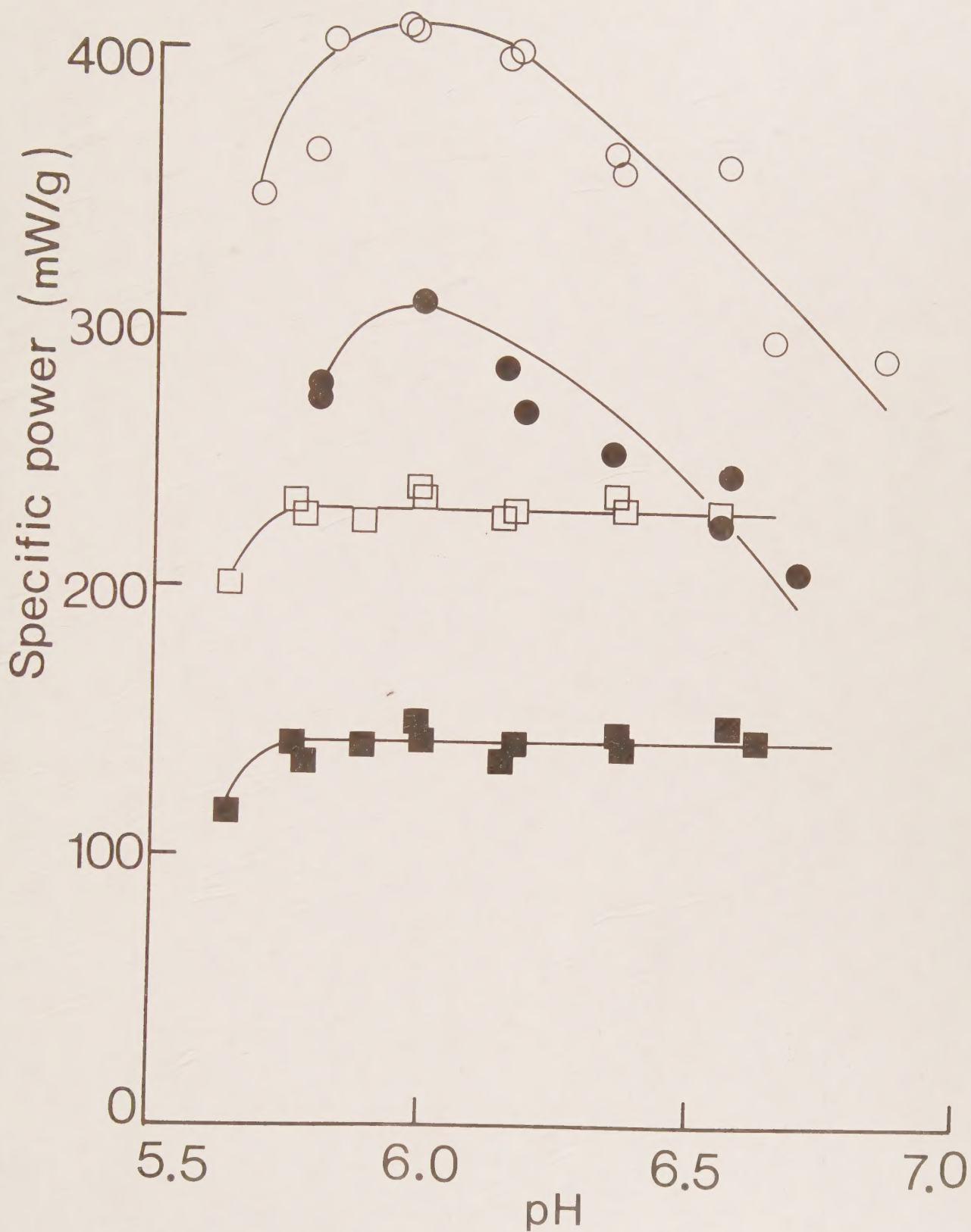


Fig 6



